INFECTION AND IMMUNITY, Feb. 2000, p. 535–542 Vol. 68, No. 2
0019-9567/00/$04.00+0

Nonpolar Inactivation of the Hypervariable Streptococcal Inhibitor of Complement Gene (*sic*) in Serotype M1 *Streptococcus pyogenes* Significantly Decreases Mouse Mucosal Colonization

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Received 3 September 1999/Returned for modification 6 October 1999/Accepted 29 October 1999

Group A *Streptococcus* (GAS) is a human pathogen that commonly infects the upper respiratory tract. GAS serotype M1 strains are frequently isolated from human infections and contain the gene encoding the hypervariable streptococcal inhibitor of complement protein (Sic). It was recently shown that Sic variants were rapidly selected on mucosal surfaces in epidemic waves caused by M1 strains, an observation suggesting that Sic participates in host-pathogen interactions on the mucosal surface (N. P. Hoe, K. Nakashima, S. Lukomski, D. Grigsby, M. Liu, P. Kordari, S.-J. Dou, X. Pan, J. Vuopio-Varkila, S. Salmelinna, A. McGeer, D. E. Low, B. Schwartz, A. Schuchat, S. Naidich, D. De Lorenzo, Y.-X. Fu, and J. M. Musser, Nat. Med. 5:924–929, 1999). To test this idea, a new nonpolar mutagenesis method employing a spectinomycin resistance cassette was used to inactivate the *sic* gene in an M1 GAS strain. The isogenic Sic-negative mutant strain was significantly ($P < 0.019$) impaired in ability to colonize the mouse mucosal surface after intranasal infection. These results support the hypothesis that the predominance of M1 strains in human infections is related, in part, to a Sic-mediated enhanced colonization ability.

Group A *Streptococcus* (GAS) is a gram-positive bacterial pathogen that causes human disease globally. The organism is responsible for many diverse infection types, including pharyngitis, acute rheumatic fever, and invasive diseases such as bacteremia and necrotizing fasciitis. A resurgence of invasive disease episodes in recent years has been documented in many countries, including those in North America, Europe, and the Far East (reviewed in reference 23). Extensive epidemiological studies have found that in most localities, strains expressing serotype M1 protein predominate (23). The M protein is a highly polymorphic surface protein that is an important virulence factor of GAS, in part because it is antiphagocytic (8). More than 80 M protein types have been recognized on the basis of serologic studies conducted over decades and more recently DNA sequencing investigations (2). The molecular explanation for the abundant representation of serotype M1 strains among invasive isolates is unknown.

Molecular characterization of serotype M1 strains cultured from patients with invasive infections has shown that in general, there is relatively restricted genetic diversity among most strains (22, 23). For example, analysis of variation in 10 genes in large samples of M1 GAS from several countries failed to identify sequence diversity in virtually all genes studied (22, 23). Although most M1 strains are closely related in overall genomic character, recently it has been shown that a protein that inhibits the normal cytolytic effect of the C5b-C9 membrane attack complex of human complement is remarkably hypervariable (1, 10, 29). This protein, known as the streptococcal inhibitor of complement (Sic), is encoded by the *sic* gene that is located in the Mga virulence regulon of GAS (1, 14). Initial DNA sequence analysis of *sic* variation among 165 M1 strains identified 62 alleles, virtually all of which would encode distinct protein variants (29). The observation that most gene alleles would encode distinct protein variants, together with a lack of synonymous (silent, not resulting in amino acid replacements) nucleotide substitutions strongly suggested that the Sic protein is under strong natural selection pressure. This idea is supported by the preponderance of radical amino acid replacements (polar-nonpolar replacements or those resulting in charge changes) found among the Sic variants (10, 29).

Serotype M1 strains also have the ability to undergo rapid changes in disease frequency and severity (19, 21, 23). Epidemic waves of serotype M1 strains have been documented in several countries (23). Although the exact molecular explanation for the ability of M1 strains to cause epidemic waves is unknown, recent analysis of the molecular population genetics of epidemic waves of serotype M1 organisms in Finland; Ontario, Canada; and four regions in the United States found that in contrast to the prevailing idea that these epidemic waves are mono- or pauciclonal, they are composed of M1 strains with a highly diverse array of Sic variants that are rapidly selected in the course of the epidemic waves (10). Study of M1 strains from humans with pharyngitis and analysis of organisms recovered from the nasopharynx of persistently colonized mice discovered that new Sic variants are selected on the mucosal surface.

Taken together, the data suggest that Sic participates in

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristics	Source or reference
Strains		
GAS		
MGAS 5005	*sic1.01*; wild-type strain	J. Vuopio-Varkila
MGAS 5005 *sic*	*sic*-inactivated isogenic mutant strain	This study
MGAS 1600	*sic1.07*; wild-type strain	K. Johnston
MGAS 1600 *sic*1	*sic*::spc1; in-frame insertion	This study
MGAS 1600 *sic*2	*sic*::spc2; out-of-frame insertion	This study
E. coli XL1-Blue	Cloning strain	Stratagene
Plasmids		
pBC KS$^-$	Cm^r; *E. coli* cloning vector	Stratagene
pEU904	Cm^r Sp^r; source of *aad* gene	J. R. Scott
pSL60-1 to -3	Cm^r (Sp^r)[a]; spc1- to 3 cassettes in pBC KS$^-$	This study
pFW12	Sp^r; *E. coli* cloning vector	27
pFW14	Cm^r; source of *cat* gene	27
pLEX5B	Ap^r; *ori* ColE1; high-copy-number *E. coli* vector	6
pSL97	Ap^r; *ori*R1; low-copy-number *E. coli* vector	This study
pSL99	Ap^r; *ori*R1; *sic1.07*	This study
pSL114	Cm^r; *ori*R1; *sic1.07*	This study
pSL123	Sp^r; 3′ flanking region of *sic1.01* in pFW12	This study
pSL124	Sp^r; 5′ flanking region of *sic1.01* in pSL123	This study
pSL127	Sp^r; *sic1.01*::spc2 in pSL124	This study
pSL128-1	Cm^r; *ori*R1; *sic1.07*::spc1	This study
pSL128-2	Cm^r; *ori*R1; *sic1.07*::spc2	This study

[a] (Sp^r), spectinomycin resistance upon induction with IPTG.

host-pathogen interaction on the mucosal surface. To directly test this idea, we constructed an M1 isogenic mutant strain in which the *sic* gene was inactivated by a new nonpolar mutagenesis strategy. The nonpolar mutagenesis approach was necessary because the *sic* gene is located in the Mga regulon and hence is located close to genes encoding important GAS virulence factors, including *emm* and *scpA*, encoding M1 protein and a protease that cleaves and inactivates complement factor C5a, respectively. The isogenic mutant strain had significantly decreased ability to colonize the nasopharynx of mice. We hypothesize that the predominance of M1 strains in human infections is related, in part, to a Sic-mediated enhanced colonization ability.

MATERIALS AND METHODS

Bacterial strains and plasmids. The bacterial strains and plasmids used in this study are described in Table 1. GAS strain MGAS 1600 was used for cloning the *sic1.07* allele, which contains a naturally occurring T→C mutation located in the −10 region of the presumed *sic* promoter. This mutation is associated with decreased Sic expression by the source strain. The strain (MGAS 5005) used to generate the isogenic Sic-deficient mutant contains the most frequently identified *sic* allele (*sic1.01*) and produces abundant levels of secreted Sic protein in vitro. This strain also has the *emm1.0* gene allele and genes encoding SpeA and SpeC exotoxins.

GAS strains were grown in Todd-Hewitt broth (Difco Laboratories, Detroit, Mich.) supplemented with 0.2% yeast extract (THY medium). Brain-heart infusion agar (Difco Laboratories) or tryptose agar with 5% sheep blood (Becton Dickinson, Cockeysville, Md.) was used as solid medium. For mutant selection, brain heart infusion agar supplemented with spectinomycin (150 μg/ml) was used. The GAS strains were incubated at 37°C in 5% CO_2–20% O_2 atmosphere.

Cloning experiments were performed with *Escherichia coli* XL-1 Blue (Stratagene, La Jolla, Calif.).

Construction of nonpolar spc cassettes. A nonpolar spectinomycin resistance (spc) cassette was developed to inactivate the *sic* gene (Fig. 1A). The cassette contains the promoterless spectinomycin resistance marker encoded by the *aad* gene (16). The 5′ region of the cassette upstream of the *aad* gene has stop codons in all three reading frames. A consensus ribosome-binding site (GGAGG) followed by the ATG start codon is located at the 3′ end. To avoid a polarity effect on downstream genes, the spc cassette is cloned so that the ATG start codon is in frame with the downstream mutated gene, in this case *sic*. Construction of the three cassettes allows one of them to be used at any restriction site available in the target gene. This strategy was based on similar *aph*-3 (20) and *cat* (chloramphenicol resistance) (17) cassettes used successfully for nonpolar gene inactivation in gram-negative bacteria.

The *aad* gene contained on plasmid pEU904 (provided by J. R. Scott, Emory University) and synthetic regulatory sequences described above which flank the *aad* gene were amplified by PCR with three sets of primers. One forward primer (spcF [GGACCCGGGTGACTAAATAGTGAGGAGGATATATTTG]) complementary to the 5′ region of the *aad* gene and three different reverse primers (spcR1 [CCTCCCGGGCATGTGATTTTCCTCCTTTTTATAATTTTTTTAA TCTGTTA], spcR2 [CCTCCCGGGCCATGTGATTTTCCTCCTTTTTATAA], and spcR3 [CCTCCCGGGTCCATGTGATTTTCCTCCTTTTTATAA]) were used. These three PCR products are flanked by *Sma*I sites and were back cloned into the *E. coli* vector pBC KS$^-$ (Stratagene) to obtain cassettes spc1 to 3, contained on plasmids pSL60-1 to -3, respectively (Table 1). The sequences of the spc cassettes were confirmed to rule out spurious mutations. All three cassettes conferred spectinomycin resistance to *E. coli* in the presence of isopropyl-β-D-thiogalactopyranoside (IPTG).

spc cassettes are nonpolar in GAS: proof of principle. To test the utility of the nonpolar mutagenesis strategy, two *sic* mutants were generated with the spc1 and spc2 cassettes by allelic replacement in the chromosome of GAS. Initial data indicated that *sic* cloned into *E. coli* was unstable, perhaps due to a toxic effect of the Sic protein. Hence, a strain (MGAS 1600) with a mutation in the −10 region of the presumed *sic* promoter that is associated with decreased Sic production by the parental GAS strain was used as the source organism for the cloned *sic* allele (*sic1.07*). The strategy for the cloning of the *sic1.07* gene and insertion of the spc1 and spc2 cassettes is shown in Fig. 1B and C. The *sic1.07* allele was amplified with *sic*-specific primers (*sic*1 [TAAGGAGAGGTCACAA ACTA] and *sic*2 [TTACGTTGCTGATGGTGTAT]) and cloned in a promoterless low-copy-number plasmid vector pSL97 (Table 1). This vector was based on plasmid pLEX5B (6), in which the ColE1 origin of replication (*ori*) was replaced with a temperature-sensitive *ori*R1 which allows the plasmid to be maintained at low copy number (15). Next, the β-lactamase gene was replaced with the *cat* gene from pFW14 (27), resulting in plasmid pSL114; plasmid pSL114 cannot replicate in GAS. This cloned *sic1.07* allele was then interrupted by insertion of the spc1 and spc2 cassettes after the sixth base pair of the *sic* coding sequence (Fig. 1C). The spc1 cassette was inserted in frame with the downstream *sic* region (pSL128-1), whereas the spc2 cassette insertion (pSL128-2) was out of frame. These two constructs were used to generate the *sic* mutants of MGAS 1600 by allelic replacement (Fig. 2A).

Since insertion in the *sic*::spc1 mutant resulted in only one amino acid change in the signal peptide of the Sic protein, production of secreted Sic by this mutant was expected. In contrast, the out-of-frame *sic*::spc2 insertion should cause early translation termination, thereby resulting in lack of Sic secretion. As expected, the in-frame mutant (MGAS 1600 *sic*1 [*sic*::spc1]) produced extracellular Sic, whereas the out-of-frame mutant (MGAS 1600 *sic*2 [*sic*::spc2]) did not (Fig. 2B). These results indicated that the spc cassettes can be used successfully for nonpolar mutagenesis in GAS.

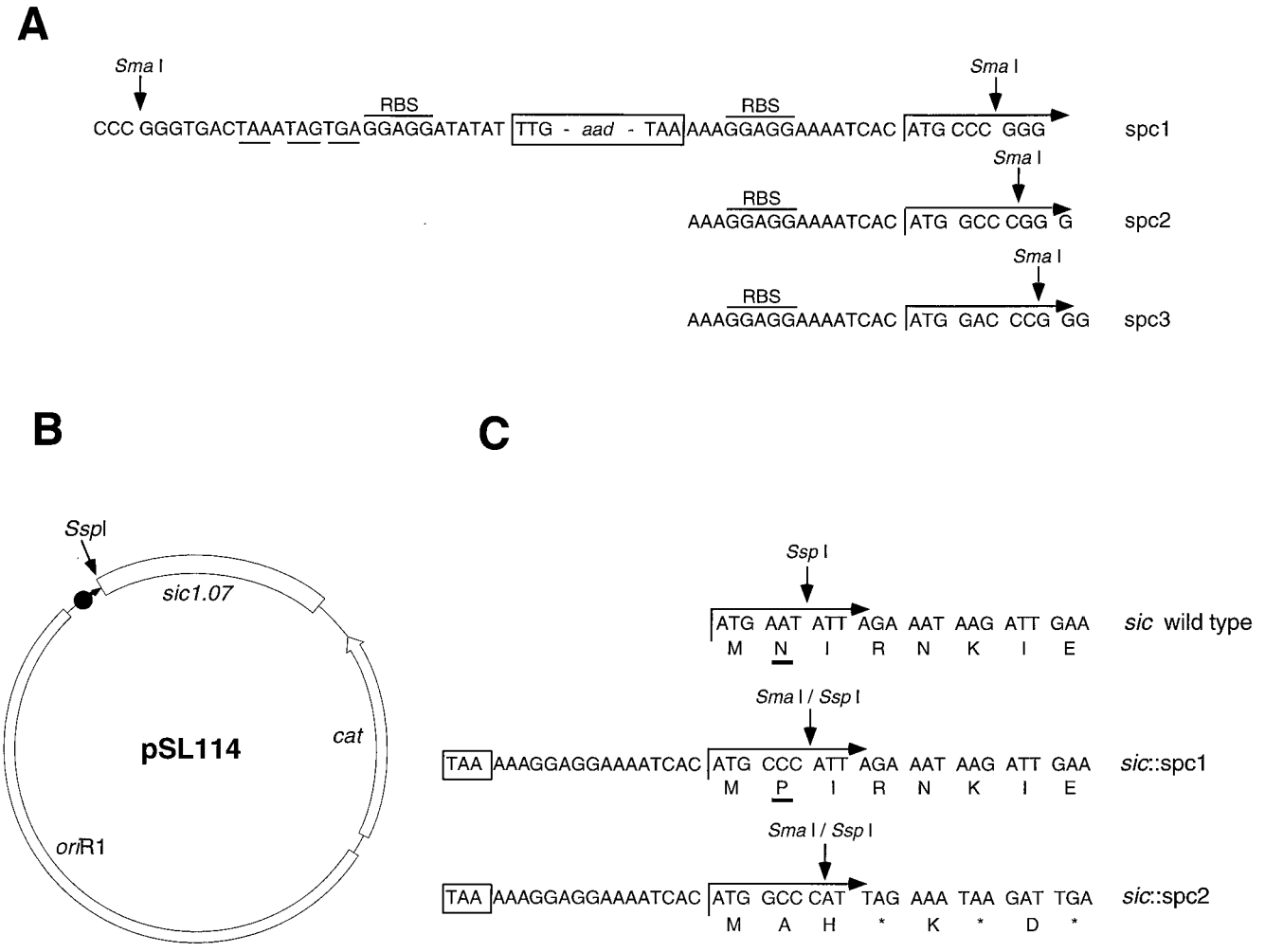

FIG. 1. Construction and testing of the spc cassettes. (A) Schematic representation of the promoterless spc cassettes. spc1- to 3 cassettes are flanked by *Sma*I sites and contain the *aad* gene conferring resistance to spectinomycin. The promoterless *aad* gene was amplified with its original ribosome-binding site (RBS). At the 5′ synthetic end, upstream of the *aad* gene, there are stop codons in all three reading frames (underlined). At the 3′ synthetic end, downstream of the *aad* gene, the spc1- to 3 cassettes contain the GGAGG consensus ribosome-binding sites and the ATG start codons in all three reading frames. (B) Construction of the suicide plasmid pSL114 containing *sic1.07*. pSL114 has a temperature-sensitive origin of replication (*ori*R1) which maintains low copy number at 30°C. It carries the *cat* gene. (C) Construction of the polar and nonpolar mutations within *sic1.07*. The first eight amino acids of the Sic signal peptide are shown. The *Ssp*I cleaves in frame after the sixth base pair of the wild-type *sic* sequence. The spc1 and spc2 cassettes were cloned at the *Ssp*I site of pSL114, resulting in plasmids pSL128-1 and 128-2, respectively. After passing the TAA stop codon (boxed) of the *aad* gene, translation is restored at the ATG start codon located at the 3′ end of the cassettes. An insertion of the spc1 cassette is in frame and results in a single amino acid N→P (underlined) substitution within the Sic signal peptide. An out-of-frame insertion of the spc2 cassette generates multiple translational stop codons (asterisks).

Construction of the *sic*-inactivated isogenic mutant strain. An analogous strategy was used to generate a nonpolar *sic1.01* knockout isogenic strain of MGAS 5005. We selected the *sic1.01* allele for inactivation because this is the most common *sic* allele found in clinical isolates of GAS serotype M1. For example, the *sic1.01* allele was found in 36% of M1 GAS isolates recovered in Finland from 1988 to 1998 (10). Suicide plasmid pSL127 was constructed (detailed cloning strategy is shown in Fig. 3) such that an internal part of the *sic* gene (bp 6 to 811) was replaced with the spc2 cassette (in-frame insertion). This construct was introduced by electroporation into the wild-type MGAS 5005 organism to obtain a nonpolar isogenic *sic*-inactivated mutant derivative. After initial screening, the MGAS 5005 *sic* strain was analyzed by PCR, Southern and Western blotting, and sequencing.

DNA methods. Standard methods (28) were used to manipulate DNA. Plasmid DNA was prepared with a QIAGEN-tip (Qiagen, Valencia, Calif.). Chromosomal DNA was isolated from GAS as described previously (22); 5 to 6 μg was used for Southern blotting. Hybridization was performed with a nonradioactive labeling and detection system (ECL [enhanced chemiluminescence]; Amersham Pharmacia Biotech, Inc., Piscataway, N.J.). DNA amplification was performed with *Taq* polymerase (Perkin-Elmer). When PCR was performed directly from *E. coli* or GAS colonies, cells were transferred to the reaction mixture on a disposable tip. The *Taq* DyeDeoxy terminator cycle sequencing kit (Applied Biosystems, Inc., Foster City, Calif.) and an ABI 377 instrument were used to obtain DNA sequence data.

RNA methods. Total RNA was prepared from GAS cultures (10 ml) grown in THY medium to mid-log phase (optical density at 600 nm [OD_{600}] of ~0.4). Cell pellets were suspended in 500 μl of TE buffer (10 mM Tris [pH 7.0], 1 mM EDTA) and treated at 37°C for 5 min with 6 μl of mutanolysin (1 mg/ml), 60 μl of lysozyme (10 mg/ml), and 25 μl of the RNase inhibitor aurintricarboxylic acid (100 mM stock solution; Sigma, St. Louis, Mo.). GAS cells were lysed with 60 μl of 20% sodium dodecyl sulfate and 600 μl of acid-phenol–chloroform (5:1 mix, pH 4.5; Ambion, Austin, Tex.) at 65°C for 5 min. Samples were extracted with equal volumes of acid-phenol–chloroform until no debris was seen at the interface. The RNA contained in 400 μl was mixed with 15 μl of 5 M NaCl and precipitated with 2 volumes of ethanol. DNA was removed by DNase I digestion followed by an acid-phenol–chloroform extraction. The RNA was precipitated as described above. Usually, 250 to 300 μg of total RNA was obtained from a 10-ml culture by this method. The RNA showed good sample integrity when analyzed in an agarose gel.

Samples (15 μg) of total RNA were denatured and separated in a 0.8% formaldehyde gel, blotted to a Nytran SuPerCharge membrane (Schleicher & Schuell, Keene, N.H.), and cross-linked. RNA transfer, hybridization (42°C), and posthybridization washes were performed with NorthernMax reagents (Am-

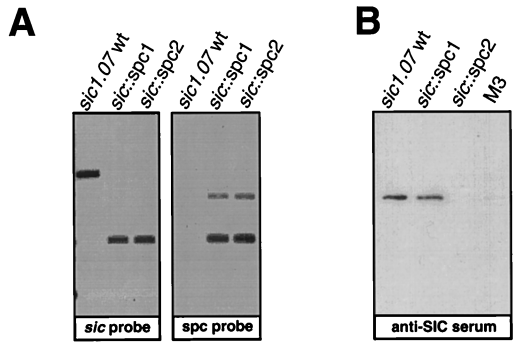

FIG. 2. Nonpolar mutagenesis of the *sic* gene by using the spc cassette. (A) Southern blot analysis of the genomic DNAs isolated from the MGAS 1600 (*sic1.07*) wild-type strain and from MGAS 1600 *sic*1 (*sic*::spc1, in-frame insertion) and MGAS 1600 *sic*2 (*sic*::spc2, out-of-frame insertion) mutants. DNAs were digested with *Ban*II, which has a single recognition site within the *aad* gene of the spc cassettes but not in the *sic* sequence. Only one mutated *sic* copy can be detected in the mutant strains by using the *sic*-specific probe homologous to the 3′ end of the *sic* gene. As expected, the spc probe hybridized only to the DNA of the mutants. (B) Immunoblot detection of the Sic protein in GAS culture supernatants. Sic1.07 was produced by the MGAS 1600 wild-type strain and by the in-frame *sic*::spc1 mutant. No Sic could be detected in the culture supernatant of the *sic*::spc2 out-of-frame mutant. The identically processed culture supernatant of a GAS serotype M3 strain lacking the *sic* gene is shown as a negative control.

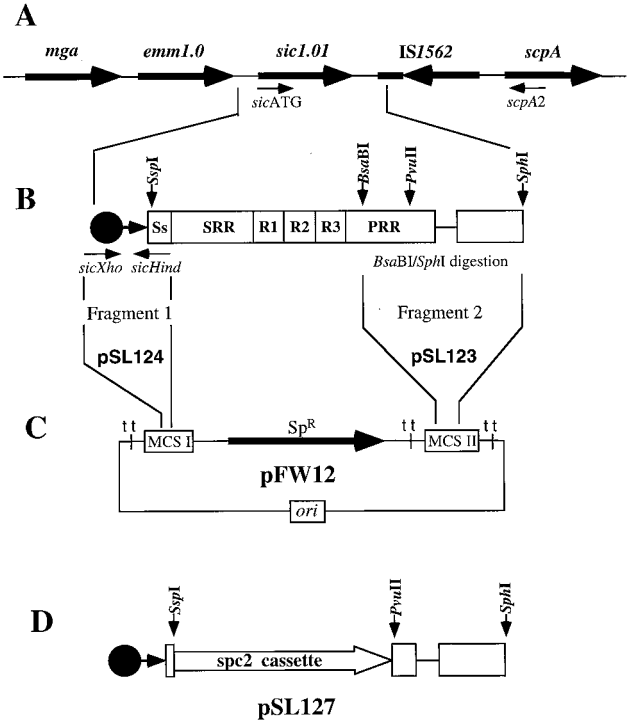

FIG. 3. Construction of the suicide plasmid pSL127, used to generate an *sic1.01*-inactivated MGAS 5005. All primers and restriction sites used in the cloning strategy are shown. (A) Schematic representation of the Mga regulon with the *sic* gene in MGAS 5005 (not to scale). The map was drawn based on PCR analyses using different combinations of primers specific for the *emm1.0*, *sic1.01*, IS*1562*, and *scpA* genes. (B) DNA region containing the *sic* gene and part of the IS*1562* used in plasmid construction. Major parts of the *sic* sequence are shown: Ss, signal sequence; SRR, short repeat region; R1 to R3, repeat regions 1 to 3; PRR, proline-rich region. Two chromosomal fragments from MGAS 5005 were amplified. Fragment 1 (~150 bp), flanked by the PCR-generated *Xho*I (*sicXho* [TCGACTCGAGGTTAAGGAGAGGTCAC]) and *Hin*dIII (*sicHind* [TTTTCAAGCTTATTTCTAATATTC]) sites, contained the *sic* promoter region and first 26 bp of the coding region. Fragment 2 (~830 bp), obtained by restriction digestion of the PCR product (*sic*ATG [GGAGAGAATACTAATG AATATTAG] and *scpA2* [CTGGTGTATCAGCAGTTTTAGC]) with restriction enzymes *Bsa*BI and *Sph*I, contained a 3′ end of the *sic* proline-rich region and adjusted part of IS*1562*. (C) First, fragment 2 was cloned into multiple cloning site (MCS) II of the *E. coli* vector pFW12, generating plasmid pSL123. Next, fragment 1 was cloned into MCS I of the pSL123, resulting in construct pSL124. (D) In the last step, the nonpolar spc2 cassette was cloned in frame between the *Ssp*I and *Pvu*II sites of the *sic* coding sequence, replacing the original spectinomycin resistance marker (Sp^R) of the vector. In this plasmid, designated pSL127, the spc2 insertion limits the amount of the *sic* coding region to 6 bp (2 amino acids) at the 5′ end and 129 bp (43 residues) at the 3′ end. Suicide plasmid pSL127 was used to generate an *sic*-inactivated isogenic variant of MGAS 5005.

bion). Biotinylated molecular weight markers (Millenium; Ambion) were used to evaluate transcript sizes.

Genomic DNA purified from the homologous strain (MGAS 5005) was used to amplify DNA probes with primers specific for the *emm* (9), *sic* (1), *scpA* (4), and *recA* (30) genes (Table 2). DNA probes (500 to 600 bp) corresponding to gene regions encoding the amino-terminal parts of the mature proteins were biotinylated with BrightStar labeling reagents (Ambion). Hybridizing bands were detected with streptavidin-POD conjugate (Roche Molecular Biochemicals, Indianapolis, Ind.) and visualized with an ECL reagent.

The transcripts were quantitated by densitometry with the BioImage Whole Band Analysis computer program (BioImage, Ann Arbor, Mich.). The amounts of the transcripts were normalized by comparison with the amount of the *recA* transcript in the same sample.

Protein methods. Sic expression was assayed in the culture supernatants of GAS grown to exponential phase (OD_{600} of ~0.5) in THY medium. Proteins were precipitated with 30% ammonium sulfate and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis followed by staining with Coomassie brilliant blue or by Western blotting with rabbit polyclonal serum raised against purified Sic1.01 (10). An ECL visualization protocol was used.

Measurement of anti-Sic immunoglobulin G (IgG) in mouse and human sera. Mouse sera were obtained on day 24 from the blood of 34 animals that survived 21 days after intranasal inoculation with the MGAS 5005 wild-type strain. Eleven mice were persistently colonized in the throat with GAS, and 23 were not. Serum samples from 15 of the mice that were not colonized on day 24 and all 11 samples from persistently colonized mice were assayed. Human sera from 198 healthy individuals with no history of invasive GAS disease were used for the analysis.

For detection of anti-Sic1.01 IgG in mouse and human sera, plate wells were coated with purified Sic1.01 (10 μg/ml in carbonate buffer, pH 9.6) at room temperature overnight. The wells were washed three times with phosphate-buffered saline (PBS) containing 0.1% Tween 20 (TPBS) and blocked with 5% gelatin in TPBS. For detection of anti-Sic IgG in mouse sera, twofold dilutions of each serum sample were prepared in PBS from a starting dilution of 1:20. One hundred microliters of each dilution was added to test wells. Mouse serum that did not react with Sic1.01 was used as a negative control. The plate blank consisted of 100 μl of PBS. For detection of anti-Sic IgG in human sera, microtiter plates were coated with Sic1.01 and washed as described above. Human sera were used at a single dilution of 1:200. Human sera that did not react with Sic1.01 were used as negative controls. The plate blank consisted of 100 μl of PBS. For both mouse and human sera, the plates were incubated at 37°C for 2 h. The plates were washed three times with TPBS, and 100 μl of horseradish peroxidase-conjugated goat antibody directed against mouse or human IgG (whole molecule, 1:2,000; Bio-Rad, Hercules, Calif.) was added to all wells. The plates were then incubated for 2 h at 37°C, washed three times as described above, and incubated with 2,2′-azino-bis-[3-ethylbenzthiazoline-6-sulfonic acid] (ABTS; Roche Molecular Biochemicals) as the development agent for 20 min at room temperature in the dark. Absorbance was measured at 405 nm with a Spectramax PLUS instrument (Molecular Dynamics, Sunnyvale, Calif.). Titers of anti-Sic IgG in mouse sera were calculated with SOFTmax PRO software version 2.6.1. Human serum samples were considered to have specific Sic antibody if absorbance readings were greater than the average of the negative controls (i.e., human sera that did not react with Sic1.01) plus 3 standard deviation values.

Mouse colonization experiments. Isogenic GAS wild-type and *sic* mutant strains were grown in THY medium and harvested in the logarithmic phase (OD_{600} of ~0.5 to 0.6), when abundant Sic production occurs. The cells were washed once and resuspended in sterile ice-cold, pyrogen-free PBS to give the required inoculum. Colony counts were performed to determine the actual number of CFU used in each experiment. Control animals inoculated with PBS were always culture negative for GAS. The animals were anesthetized by inhalation of Metofane (Mallinckrodt Veterinary, Mundelein, Ill.) prior to experimental procedures.

Mouse throat colonization studies were conducted with adult (18- to 20-g) male outbred CD-1 Swiss mice (Harlan Sprague-Dawley Inc., Houston, Tex.) as described previously (18). The mice were inoculated in the nares with 50 μl of GAS cell suspensions. The mouse throats were swabbed periodically for 3 weeks. GAS cells present on swabs were suspended in 1 ml of PBS, and 0.1 ml from these samples was cultured on blood agar. Blood was collected from dead

TABLE 2. Primers used to amplify DNA probes for Northern hybridization

Gene	Primer designation	Primer sequence	Probe size (bp)	Reference
emm	*emm*For	CACGAATAGACACTATTCGCTTAG	629	9
	*emm*Rev	CAAGTTCAAGTTTTGCATTGCC		
sic	*sic*For	CCGAAACGTATACATCACGC	494	1
	*sic*Rev	CTCAGGTTTAGGAATATGTCC		
scpA	*scpA*For	CTGTGACAGAAGACACTCC	500	4
	*scpA*Rev	CCCTGACACGTGTGTGCC		
rec	*rec*For	CGTTCAGGAAGTCTAGCTC	553	30
	*rec*Rev	CATCCAGCCGAACAGAAGC		

animals by cardiac puncture and cultured on blood agar. All blood samples from dead animals grew GAS. In addition, *sic* mutant colonies recovered from both dead and colonized mice were tested for reversion by streaking on medium with spectinomycin. All 400 colonies tested (one-half isolated from the blood and one-half from the throat) maintained the spectinomycin-resistant phenotype, indicating that the mutant was stable in the host without antibiotic selection.

Statistical analysis. Fisher's exact two-tailed test was used to assess statistical differences in mouse mortality and throat colonization between the animal groups infected with the wild-type GAS or the isogenic *sic* mutant strain. A logistic regression model was used to compare mouse throat colonization by the wild-type and mutant strains. Statistical calculations were performed with SAS software (SAS Institute Inc., Cary, N.C.).

RESULTS

Anti-Sic immune response in mice and humans. Recent investigation discovered that in contrast to other genes analyzed (including *emm1*, encoding M1 protein), the *sic* gene and Sic protein are highly variable among serotype M1 GAS recovered in epidemic waves in all geographic localities studied (10). In addition, Sic structural variants were selected after intranasal inoculation of mice but not during in vitro cultivation (10, 29). These observations imply that Sic is made in vivo, but this aspect of GAS pathogenesis has not been studied. If Sic is made in vivo, we expected that infected mammalian hosts would make antibody against this protein. To test this issue, we first analyzed the anti-Sic serum antibody response in mice inoculated intranasally with wild-type MGAS 5005. Nine of 11 animals (82%) persistently colonized in the throat on day 24 after intranasal inoculation had specific anti-Sic1.01 IgG antibodies as assessed by enzyme-linked immunosorbent assay (ELISA) (Fig. 4). In contrast, animals that were not persistently colonized after intranasal inoculation lacked anti-Sic serum antibodies.

These data suggested that Sic was expressed in the course of GAS infection of the human mucosal surface. To address this possibility, we tested serum obtained from 198 randomly selected children between 6 months and 18 years of age for anti-Sic serum IgG by ELISA. These children had no known history of invasive GAS disease. Approximately one-fourth (46 of 198 [23%]) has serum antibody against Sic, a result indicating that this protein is also made during human mucosal infection. Detailed analysis of the human serologic response to Sic will be presented elsewhere.

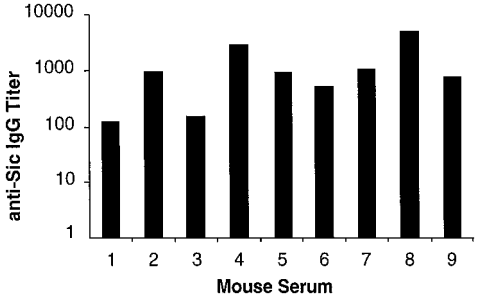

FIG. 4. Graph of anti-Sic1.01 IgG titers in serum from mice persistently colonized in the throat with MGAS 5005. Serum samples were obtained on day 24 postinfection and tested in an ELISA against purified Sic1.01 at a starting dilution of 1:20. Titers were calculated with SOFTmax PRO software version 2.6.1.

Construction and characterization of the Sic1.01-negative nonpolar isogenic mutant. The presence of anti-Sic antibody in experimental mice and humans indicated that Sic was expressed during mucosal infection. In addition, interaction with the host mucosal surface has been shown to result in a high frequency of selection of new Sic variants (10). Inasmuch as both seroconversion to Sic and selection of Sic variants have been observed in mice and humans, we investigated if Sic participates in mouse throat colonization. A nonpolar *sic*-inactivated isogenic mutant strain was constructed to test this idea.

Strain MGAS 5005 contains the most frequently occurring *sic1.01* gene allele (10, 29) and was used to generate an isogenic *sic*-inactivated mutant derivative. This strain also has the *emm1.0* allele and genes encoding SpeA and SpeC exotoxins. To generate the Sic-negative nonpolar isogenic strain of MGAS 5005, an internal part of the cloned *sic* gene located between bp 6 to 811 was replaced with the spc2 cassette (in-frame insertional replacement). After allelic replacement, the MGAS 5005 *sic* mutant was confirmed by PCR, Southern blotting, and sequencing. Since insertion of the spc2 cassette removed most of the *sic* coding sequence (270 of the 313 amino acid residues), the mutant strain did not produce extracellular Sic protein (data not shown).

To confirm that *sic* inactivation did not have a polar effect on flanking genes, expression of the *emm* and *scpA* genes was studied (Fig. 5). This analysis was particularly important since both genes encode documented GAS virulence factors. Single transcripts of the predicted molecular size were identified for *emm*, *sic*, and *scpA* genes in the wild-type MGAS 5005 organism. No *sic*-specific transcript was detected in the MGAS 5005 *sic* mutant. In addition, there was no difference in the amount of the *emm*-specific transcripts made by the wild-type and *sic* mutant strains. Moreover, there was no significant difference in the amount of the *scpA*-specific transcript quantified in RNA samples isolated from both strains in three independent experiments. Hence, the genetic strategy ablated Sic production without altering expression of the genes flanking *sic*. *sic* inactivation did not alter the growth rate or colony morphology of the mutant (data not shown).

***sic* inactivation decreases mouse throat colonization.** To test the hypothesis that inactivation of *sic* altered mouse throat colonization, we used a logistic regression analysis to compare colonization by the wild-type GAS serotype M1 and the isogenic *sic*-inactivated mutant (Fig. 6). Five dilutions (twofold dilutions ranging from $\sim 8 \times 10^7$ to 0.5×10^7 CFU) of the

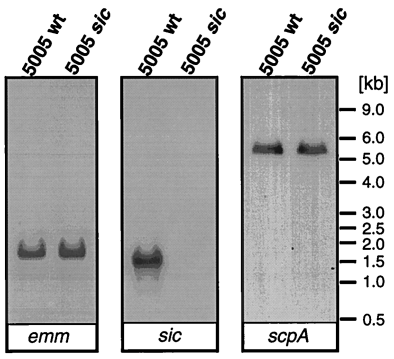

FIG. 5. Northern blot analysis of *emm*, *sic*, and *scpA* gene expression by wild-type strain MGAS 5005 (5005 wt) and by the isogenic 5005 *sic* mutant. No *sic* transcript could be detected in the mutant strain. In addition, transcription of the *emm* and *scpA* genes was unaffected. Biotinylated DNA probes were used for hybridization. The biotinylated molecular weight RNA marker was used to evaluate transcript sizes.

wild-type and mutant strains were used to inoculate groups of 10 mice intranasally. Mouse throats were swabbed periodically, and the bacteria were cultured on a blood agar. In addition, blood samples from dead animals were cultured. There was no significant difference in the number of dead mice or Kaplan-Meier survival curves on day 21 after infection with the wild-type (16 of 50 mice on day 21) or mutant (14 of 50 mice) strain. In contrast, we observed a significant difference in colonization of the mouse mucosa. Significantly more mice inoculated with the wild-type GAS strain were culture positive than mice infected with the *sic* mutant strain ($P < 0.019$) (Fig. 5). The results of a logistic regression model using GAS colonization data obtained from only the mice which survived were also significantly different ($P = 0.023$), as observed with the data obtained from the model which included dead mice (analysis described above). The odds ratio for the wild-type strain was 2.81, which indicated that the MGAS 5005 parental organism had a much higher incidence of throat colonization. However, the level of inoculum was not significant, indicating that the incidence of throat colonization was not related to the amount of inoculum given to the mice. Therefore, experiments with animals infected with all five inocula of the wild-type strain or the *sic* mutant were combined. A significant group difference

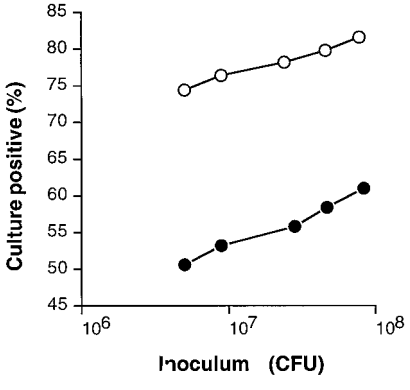

FIG. 6. Logistic regression analysis of death and throat colonization at 72 h after intranasal inoculation with the 5005 wild-type strain (open circles) or with the 5005 *sic* mutant (solid circles). Each data point represents 10 mice. The *y* axis represents estimated percent culture-positive animals. The data fit the model ($P = 0.021$); the group difference was significant ($P < 0.019$); the dose-dependent parameter was not significant.

was found ($P < 0.035$; Fisher's exact two-tailed test). We note that the difference in colonization by the wild-type and mutant strains was statistically significant during the first 4 days post-inoculation. These results suggested that Sic plays a significant role at the early stages of infection. Because the large number of the mice died during the study period, the number of surviving animals in each experimental group was not adequate for statistical comparison. Taken together, our results indicate that Sic participated in GAS-host interactions by enhancing early colonization of the mucosal surfaces.

DISCUSSION

GAS causes epidemic waves of pharyngitis and severe invasive infections worldwide (23). GAS strains of serotype M1 have been predominantly responsible for countrywide epidemics for reasons that are not known. The observations that the Sic protein was hypervariable in serotype M1 strains recovered from epidemic waves and that new structural Sic variants were selected on mucosal surfaces in the course of epidemic waves suggested that Sic could contribute to host colonization (10).

We constructed a *sic*-inactivated nonpolar isogenic mutant strain to directly assess the contribution of Sic to mucosal colonization of a mammalian host. Construction of gene-inactivated GAS isogenic strains has mainly used the conjugative transposon Tn*916* (3, 24, 32) or plasmid-directed insertional mutagenesis (25, 26, 31). Despite many successful applications, both methods have several disadvantages, including uncontrolled chromosomal insertions resulting in the need for laborious screening, presence of foreign vector sequences in the GAS genome, or existence of two copies of the mutated gene, thereby creating potential substrates for recombination. Both conjugative transposition and plasmid-directed insertional mutagenesis have the drawback of insertion instability and potential polar effects of the integron on neighboring genes. This is an especially problematic issue in assessing the contribution of Sic to host-pathogen interactions because the gene is located in the Mga regulon containing proven virulence genes. Therefore, we developed a new strategy that used a nonpolar spc cassette to inactivate the *sic* gene. Analogous *aph*-3 and *cat* cassettes have been used successfully to generate nonpolar mutants in *Shigella flexneri* (20) and in *E. coli* (17) and *Pasteurella haemolytica* (7), respectively. Two important features of our nonpolar spc cassette are shown in this study: (i) it restores the expression of a downstream gene, and (ii) since it is promoterless, it does not alter the level of transcription of a downstream gene. In our study, we showed that insertion of the spc cassette within *sic* did not alter the transcriptional pattern of genes in the Mga regulon. Specifically, the isogenic mutant did not express a *sic* transcript, and transcription of the flanking *emm* and *scpA* genes was not affected. Hence, the data indicate that this spc cassette can be used to conduct nonpolar GAS mutagenesis.

Our study showed that inactivation of *sic* decreased colonization of the host mucosal surface. Although the molecular mechanism by which Sic contributes to throat colonization is not yet known, several possibilities can be envisioned. Akesson et al. (1) reported that in vitro, Sic binds to the human plasma components clusterin and histidine-rich glycoprotein and interferes with formation of the membrane attack complex of complement. It is possible that Sic-mediated impairment of complement function enhances survival of M1 GAS strains in the host. In this regard, we note that Ji et al. (11–13) reported that inactivation of the gene (*scpA*) encoding the peptidase that cleaves complement protein C5a reduced the capacity of

GAS to colonize the throats of mice following intranasal inoculation. Immunization of mice with purified streptococcal C5a peptidase also reduced colonization capacity. It is also possible that Sic exerts other mucosal effects that create local conditions that enhance colonization. Clearly, additional studies are required to delineate the exact mechanism of Sic action at the mucosal surfaces.

The humoral immune response induced in the host to microbial infection commonly results in clearance of the organism. Many microbes escape antibody-mediated clearance by positive selection of allelic variants with altered affinity to host antibodies. Recent results have shown that GAS strains expressing new Sic variants were selected in mice following long-term throat colonization (10), but only after a colonization time sufficient to generate an anti-Sic antibody response. In the present study we showed that 82% of the persistently colonized mice raised specific serum IgGs directed against the Sic protein. Anti-Sic antibodies were also identified in the serum of human subjects. These two observations suggest that host antibody may select new Sic variants, an idea postulated in earlier studies (10, 29).

Our results demonstrate that in a mouse model Sic contributes to colonization of the mucosal surface by serotype M1 GAS. We have also shown that colonized mice, and humans with no known history of invasive GAS disease, have anti-Sic antibody, which means that Sic is expressed in the context of mucosal interaction. The results of epidemiologic studies in the United States and Finland indicate that colonization of the human mucosal surface is a critical step in determining the frequency of occurrence of invasive episodes. For example, Cockerill et al. (5) studied an outbreak of invasive disease episodes caused by a clone of serotype M3 GAS and found that the outbreak-associated strain was the most common organism recovered from nasopharyngeal carriers in the same locality. Similarly, Muotiala et al. (21) reported that in Finland between 1988 and 1992, the proportions of serotype M1 isolates among invasive and pharyngitis isolates were identical. Taken together, the results of these studies suggest that expression of a protein capable of enhancing the rate or efficiency of GAS colonization of the host mucosal surface is likely to increase the frequency of invasive disease. In this regard, it is noteworthy that expression of the Sic protein may be restricted to relatively few clonal lineages of GAS, most notably M1 organisms (1). We believe it reasonable to hypothesize that the predominance of M1 strains in human infections and the capability of M1 strains to cause epidemic waves are related in part to Sic-mediated enhanced colonization. Additional studies are under way to test this idea.

ACKNOWLEDGMENTS

This work was supported by Public Health Service grant AI-33119 to J.M.M.

We thank J. Scott for supplying plasmid pEU904.

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Editor: E. I. Tuomanen