Shr Is a Broad-Spectrum Surface Receptor That Contributes to Adherence and Virulence in Group A Streptococcus

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Group A streptococcus (GAS), also known as Streptococcus pyogenes, is a common hemolytic pathogen that produces a range of suppurative infections and autoimmune sequelae in humans. Shr is an exported protein in GAS, which binds in vitro to hemoglobin, myoglobin, and the hemoglobin-haptoglobin complex. We previously reported that Shr is found in association with whole GAS cells and in culture supernatant. Here, we demonstrate that cell-associated Shr could not be released from the bacteria by the muralytic enzyme mutanolysin and was instead localized to the extracellular environment. In vitro binding and competition assays demonstrated that in addition to hemoprotein binding, purified Shr specifically interacts with immobilized fibronectin and laminin. The absence of typical fibronectin-binding motifs indicates that a new protein pattern is involved in the binding of Shr to the extracellular matrix. Recombinant *Lactococcus lactis* cells expressing Shr on the bacterial surface gained the ability to bind to immobilized fibronectin, suggesting that Shr can function as an adhesin. The inactivation of *shr* resulted in a 40% reduction in the attachment to human epithelial cells in comparison to the parent strain. GAS infection elicited a high titer of Shr antibodies in sera from convalescent mice, demonstrating that Shr is expressed in vivo. The *shr* mutant was attenuated for virulence in an intramuscular zebrafish model system. In summary, this study identifies Shr as being a new microbial surface component recognizing adhesive matrix molecules in GAS that mediates attachment to epithelial cells and contributes to the infection process.

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Group A streptococcus (GAS) is a common hemolytic pathogen that produces a range of suppurative infections and autoimmune sequelae in humans. Shr is an exported protein in GAS, which binds in vitro to hemoglobin, myoglobin, and the hemoglobin-haptoglobin complex. We previously reported that Shr is found in association with whole GAS cells and in culture supernatant. Here, we demonstrate that cell-associated Shr could not be released from the bacteria by the muralytic enzyme mutanolysin and was instead localized to the extracellular environment. In vitro binding and competition assays demonstrated that in addition to hemoprotein binding, purified Shr specifically interacts with immobilized fibronectin and laminin. The absence of typical fibronectin-binding motifs indicates that a new protein pattern is involved in the binding of Shr to the extracellular matrix. Recombinant *Lactococcus lactis* cells expressing Shr on the bacterial surface gained the ability to bind to immobilized fibronectin, suggesting that Shr can function as an adhesin. The inactivation of *shr* resulted in a 40% reduction in the attachment to human epithelial cells in comparison to the parent strain. GAS infection elicited a high titer of Shr antibodies in sera from convalescent mice, demonstrating that Shr is expressed in vivo. The *shr* mutant was attenuated for virulence in an intramuscular zebrafish model system. In summary, this study identifies Shr as being a new microbial surface component recognizing adhesive matrix molecules in GAS that mediates attachment to epithelial cells and contributes to the infection process.

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sential nutrients such as iron. GAS growth in vitro in iron-restricted medium is supported by whole blood, serum, myoglobin, hemoglobin, and the hemoglobin-haptoglobin complex but not by the ferric carrier proteins transferrin and lactoferrin (13, 16, 40). Hence, heme, the most abundant iron form in mammals, serves as a major source of iron for this hemolytic pathogen. The shr operon, is an iron-regulated operon in GAS involved in heme acquisition and transport. In addition to five genes with unknown function, the shr operon carries the sia operon and mediates bacterial attachment. We report that Shr is expressed in vivo and is important for GAS virulence in a zebrafish infection model.

MATERIALS AND METHODS

Bacterial strains and growth conditions. Escherichia coli strains DH5α and Top10 (Invitrogen) were used for cloning and gene expression. The clinical GAS (S. pyogenes) isolates used in this study were obtained from the Georgia Emerging Infections Program and are listed in Table 1. The other GAS strains used in this study were MGAS5005, an M type 1 strain (49); JR65, an M type 6 strain (46); NZ131, an M type 49 strain (47); and ZE492, a shr mutant constructed in NZ131 (provided by Bernard Beall, Centers for Disease Control and Prevention Respiratory Diseases Branch, Atlanta, GA) (Fig. 2). The shr mutation in ZE492 is a deletion-insertion mutation made by replacing an internal 0.3-kb BglII fragment with the spectinomycin resistance gene aadA9 (B. Beall, personal communication). We confirmed the structure of the shr mutation in ZE492 genome by sequence analysis of a DNA fragment carrying the mutant shr allele amplified from the ZE492 chromosome with primers ZE245 (5′-GTCGCCAC AAAACCAAGGCAC-3′) and ZE246 (5′-CAGTCGATGATCAGTCGCG AG-3′). Lactococcus lactis strain MG1363 was used as a heterologous host for the expression of the native Shr protein from plasmid pXLI4. E. coli cells were grown in Luria-Bertani broth with agitation. GAS was grown stably in Todd-Hewitt broth with 0.2% (wt/vol) yeast extract (THY broth; Difco Laboratories). L. lactis was grown statically at 30°C in M17 medium (Difco Laboratories) supplemented with 0.5% (wt/vol) glucose. When necessary, spectinomycin at 100 μg/ml or kanamycin at 70 μg/ml was added to the medium.

Nucleic acid methods. Chromosomal and plasmid DNA extraction and DNA manipulations including restriction digests, cloning, and DNA transformation into E. coli, GAS, or L. lactis were done according to the manufacturer’s recommendations and with standard protocols as previously described (12, 41). For RNA extraction and analysis, GAS cells were harvested at the logarithmic growth phase, and total RNA was prepared using the RiboPure-Bacteria kit (Ambion). RNA was quantified spectrophotometrically, and its integrity was examined by agarose gel electrophoresis. The absence of DNA contamination was verified by PCR. cDNA was produced with SuperScript II reverse transcriptase (Invitrogen) according to the manufacturer’s specifications. One microgram of RNA was used in the reverse transcription step, and 1/20 of the reaction mixture served as a template for 25 cycles of PCR. Primers SRAR (5′-CGTATCGTACTCGCTCA GCAG-3′) and SRAL (5′-GCGTTCAGGAGGTCTAGCTC-3′) were used in theanalysis of the recA gene, and primers 204A-Rev (5′-TCTGAAATGGCAT GACGTGTTC-3′) and 204A-Rev (5′-TCTGAAATGGCATGACGTGTTC-3′) were used for the analysis of siaA transcription.

Construction of shr-complemented strain ZE4924. To complement the shr mutation in ZE492, we cloned the native shr gene into temperature-sensitive plasmid pRS700 (33). A 4.5-kb fragment amplified from the NZ131 chromosome with primers ZE170 (5′-TTTTTTTAGATGGCTTTCGCTC-3′) and ZE174 (5′-TTTTTTATCGATCGTATCGACGTTC-3′) was used in the recA gene, and primers 204A-Rev (5′-TCTGAAATGGCATGACGTGTTC-3′) and 204A-Rev (5′-TCTGAAATGGCATGACGTGTTC-3′) were used for the analysis of siaA transcription.

![FIG. 1. Shr protein domains and cellular location. (A) Schematic representation of the Shr protein. The SMART algorithm (http://smart.embl-heidelberg.de) was used for the structural analysis of Shr. The location of protein domains (expressed as amino acid numbers) is shown. LP, leader peptide; NEAT 1, NEAT domain 1; TM, transmembrane domain. (B and C) Proteins prepared from NZ131 cells grown in THYB were analyzed by Western blotting using anti-Shr antibodies (B) or anti-M49 antibodies (C). T, total protein; CW, cell wall fraction; CM, cell membrane fraction. Full-length Shr and M49 are indicated by the arrows.](http://iai.asm.org/)

<table>
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<th>Strain</th>
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* Clinical strains obtained from the Georgia Emerging Infections Program. The M type (if available) is indicated in parentheses.

* NT, not typed.

* Immobilized GAS cells were reacted with NRS or with anti-Shr serum; antibody binding was detected with AP-conjugated secondary antibody.
FIG. 2. Successful inactivation of shr in strain ZE4912 and mutant complementation in strain ZE4924. (A) Schematic representation of the sia operon and the shr mutation in ZE4912. The shr mutation in strain ZE4912 consists of a small deletion and an insertion of the spectinomycin resistance gene aad9. Strain ZE4924 is a merodiploid strain, which carries both the mutant and the wild-type alleles of shr in the chromosome (see Materials and Methods). (B to D) RNA and proteins from wild-type strain NZ131 (lane 1), shr mutant strain ZE4912 (lane 2), and shr-complemented strain ZE4924 (lane 3) were prepared and analyzed. (B) Total RNA was extracted, loaded (1 μg/well) on an agarose gel, and examined for integrity. Reverse transcription-PCR products obtained with recA-specific primers (used to control for equal amounts of RNA template) or with siaA-specific primers were analyzed by agarose gel electrophoresis. (C) Total proteins were prepared and analyzed by Western blotting with anti-SiaA serum. (D) Proteins from the membrane fraction were analyzed by Western blotting with anti-Shr serum. Full-length Shr is indicated by the arrow.

(ZE4924), which contains both the mutant and the wild-type alleles of shr in the chromosome. Strain ZE4924 was made in ZE4912 by Campbell insertion. Plasmid pXL14 was introduced into ZE4912 cells. Clones in which pXL14 was integrated into the chromosome (by homologous recombination between the mutant shr allele in the chromosome and the wild-type copy on pXL14) were selected on kanamycin plates at 37°C (the nonpermissive temperature). The resulting chromosomal structure was verified by PCR analysis.

Antibodies. Rabbit polyclonal antibodies against Shr and rabbit anti-SiaA serum were previously described (4). Rabbit polyclonal antibodies against M protein type 49 (a killed whole-cell vaccine that was absorbed extensively to produce M49-specific antiserum) were provided by Bernard Beall (Centers for Disease Control and Prevention Respiratory Diseases Branch, Atlanta, GA). Rabbit immunoglobulin G (IgG) against GAS type-specific carbohydrates was purchased from Abcam, Inc. Rabbit antiserum for the metal-dependent repressor MtsR (5) was raised against purified MtsR protein and reacted with protein bands at the correct size in protein extract from wild-type strain NZ131 but not from mtsR mutant strain ZE4915 (data not shown). Mouse antiserum against Lactococcus lactis surface components was raised by intraperitoneal injection with 10⁶ CFU of MGI363 cells. A booster injection was given 10 days after the first injection. Antiserum was collected 10 days after the booster.

Preparation of GAS cell fractions. GAS cell fractionation was done as previously described, with minor modifications (36). Essentially, GAS was grown overnight in THY broth containing 20 mM glycine. Cells from 14-ml cultures at an optical density at 600 nm (OD₆₀₀ of 1.0) were harvested, washed with phosphate-buffered saline (PBS), and resuspended in 0.5 ml of either TEA [10 mM Tris-HCl buffer (pH 8.0), 1 mM EDTA, and 0.75 μg/ml of the protease inhibitor 4-(2-aminoethyl)-benzenesulfonyl fluoride hydrochloride (AEBSF); Roche] for the preparation of total cellular proteins or TEAR (TEA with 30% raffinose) for cellular fractionation. The cell suspensions were treated with the muramyl enzyne mutanolysin (500 U) for 1 h at 37°C to dissolve the cell wall. For total protein extract, the mutanolysin-treated cells were boiled in sample buffer for 10 min. For cell fractionation, the protoplasts were separated from the cell surface fraction by centrifugation, resuspended in TEA, and lysed by successive cycles of freezing (−80°C) and thawing (37°C). Membrane proteins were separated from the soluble intracellular proteins by centrifugation and were dissolved by boiling in sample buffer. The protein extracts were standardized with respect to the cell number in the corresponding culture and were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Shr, M protein type 49 (M49), or SiaA was detected by Western blotting as described previously (4).

Purification of rShr (His₆-Xpress-Shr). Recombinant Shr (rShr) was purified from E. coli cells harboring plasmid pCB1 as previously described (4), with the following modifications: the cells were resuspended in a solution containing 20 mM Tris (pH 8.0), 100 mM NaCl, 0.1% Triton X-100, 0.5 mg/ml lysozyme, and a protease inhibitor cocktail (Complete Mini, EDTA free; Roche) and incubated on ice for 30 min. The bacteria were then lysed by sonication, and rShr was purified over a nickel column (HisTrap HP; GE Healthcare) and subsequently desalted by use of a HiTrap desalting column (GE Healthcare). The purified protein was examined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Western blot analysis with anti-Shr antibodies and quantified by the Bradford assay (Bio-Rad).

ELISA. Three types of enzyme-linked immunosorbent assays (ELISAs) were used in the binding experiments.

(i) ELISA with immobilized bacteria. ELISA with immobilized bacteria was performed to study the availability of Shr on the bacterial cell surface. GAS or L. lactis cells from cultures grown overnight were harvested, washed with PBS, and used to coat 96-well ELISA/RIA microplates (Costar; Corning Inc.) overnight at 4°C. The supernatant was then aspirated, and the plates were washed with PBS containing 0.05% Tween 20 (PBST). After blocking with PBST and 5% skim milk for 1 h at 37°C, the wells were washed with PBST and incubated with the primary antibody for 1 h at 37°C. The microplates were next washed with PBST and incubated with secondary antibodies conjugated to alkaline phosphatase (AP) for 1 h at 37°C. The reaction was developed using p-nitrophenyl phosphate system, and the absorbance was read at 405 nm.

(ii) ELISA with immobilized ligands and purified Shr protein. ELISA with immobilized ligands and purified Shr protein was used to investigate the binding of Shr to host proteins. The microplates were coated with the ligands (25 μg/ml), washed, and blocked as described above. Purified Shr (in PBST–1% bovine...
(i) Analysis of L. lactis cell binding to immobilized ligands by ELISA. For analysis of L. lactis cell binding to immobilized ligands by ELISA, the microplates were coated with the ligands (25 μg/ml), washed, and blocked as described above. Samples of L. lactis cultures were harvested, washed with an equal volume of PBS, diluted into the desired concentrations in PBST–1% BSA, and applied above. Samples of L. lactis were coated with the ligands (25 μg/ml), washed, and blocked as described above. Samples of L. lactis cultures were harvested, washed with an equal volume of PBS, diluted into the desired concentrations in PBST–1% BSA, and applied above. Following 1 h of incubation at 37°C, the plates were washed with PBST and reacted with the L. lactis antisera. Binding was detected with secondary AP-conjugated secondary antibody as described above.

Detection of anti-Shr antibodies in convalescent mice. Mice were injected subcutaneously with 2 × 10^6 CFU of wild-type strain MGAS5005 as previously described (43) and were monitored for 27 days. The infected mice developed subcutaneous lesions, which healed over time in the surviving animals (~80% of the injected mice). On the 28th day postinoculation, the surviving mice were bled by cardiac puncture, and the serum was obtained by centrifugation. ELISA was used to determine the presence and the titer of anti-Shr antibodies in the mouse sera. Ninety-six-well ELISA microplates (Costar; Corning Inc.) were coated with 1 μg/ml purified Shr. Following coating at 4°C overnight, the plates were washed with PBST and blocked with 1% (wt/vol) BSA in PBST for 1 h at 37°C. The plates were then washed with PBST and incubated for 1 h at 37°C with mouse sera at different dilutions. Following incubation and washing, AP-conjugated anti-mouse antibodies diluted 1:1,000 in blocking buffer were added to the plates and incubated for 1 h at 37°C. Bound antibodies were detected by using the p-nitrophenyl phosphate system. Plate contents were incubated at room temperature for 30 min and read at 405 nm.

(ii) Hep-2 cell culture and adherence assay. HEp-2 cells were cultured in 24-well tissue culture plates with nutrient mixture medium (RPMI) supplemented with 10% fetal bovine serum at 37°C in an atmosphere with 5% CO2. For adherence assays, cells grown to a semiconfluent state (~10^5 cells/well) with antibiotics (2% penicillin-streptomycin; Thermo Scientific Hylenon Antibiotics) were washed with PBS and inoculated with GAS cells (10^6 CFU/well) harvested at the mid-log phase (OD_600 of 0.3 to 0.4), washed with THY, and resuspended in RPMI medium. The bacteria were incubated with the tissue cultures for 2 h at 37°C, at that time, nonadherent bacteria were removed by successive washes with PBS. To determine the number of adherent bacteria, HEp-2 cells were detached with 100 μl of 0.05% trypsin for 5 min at 37°C and lysed with 400 μl of 0.025% Triton X-100. The bacterial number in each sample was determined by plating onto THY agar plates. Each adherence experiment was performed in triplicate or quadruplicate.

Zebrafish care and virulence assays. Care and feeding of zebrafish (Danio rerio) were done according to previously reported methods (34, 54). Streptococci were cultured overnight in THY broth with 2% (wt/vol) peptone (THYP) at 25°C incubator. Infected fish were monitored for 72 h, and death was recorded in intervals of 12 h. The 50% lethal dose (LD₅₀) for infection was determined by plating onto THY agar plates. Each adherence experiment was performed in triplicate or quadruplicate.

Competitive assay in the zebrafish model. The competitive index (CI) was defined as the change in the population ratio of two strains after growth in zebrafish muscle. NZ131 (wild type) and the mutant strain (ZE4912) were cultured separately as described above. Cells from each strain were mixed in a 1:1 ratio to a final concentration of 10^6 CFU/ml. Zebrafish were infected i.m. with 10 μl of this mixture, resulting in an infectious dose of 10^6 CFU. After 24 h, muscle lesions were collected from euthanized fish and homogenized in PBS. The homogenates were plated onto THY and THY-streptomicyn plates, and the ratio of the mutant to the wild-type strain in the lesion was determined.

Statistical analysis. A Student’s t test was used to compare data sets derived from two groups to each other; a P value of ≤0.05 was considered to be significant. Analysis of variance (ANOVA) followed by the Tukey honest significant difference (HSD) post hoc test was used for multigroup comparisons; a P value of ≤0.05 was considered to be significant. Zebrafish survival data were analyzed by the method of Reed and Muench for the calculation of the LD₅₀. Kaplan-Meier plots of zebrafish survival were used to compare infections by the wild-type, mutant, and complemented GAS strains. The statistical significance was evaluated by the log rank test (18).

RESULTS

Shr is attached to the GAS cell membrane and is exposed to the extracellular environment. Shr is a large (1,275-amino-acid) protein found both in the culture supernatant and in association with whole cells (4). It has a functional leader peptide, and it has a putative transmembrane domain followed by a positively charged tail at its carboxy terminus (Fig. 1A). However, unlike proteins anchored to the surface of gram-positive bacteria, Shr lacks a cell wall-anchoring motif at its C terminus (45), suggesting that after it is exported to the cell surface, Shr may be bound to the cell membrane and not anchored to the cell wall. To determine the cellular location of Shr, GAS strain NZ131 (M type 49) was fractionated following digestion with the muraclytic enzyme mutanolysin and examined by Western blot analysis. The Shr protein (145 kDa) was not released by this process but was found in the membrane fraction instead (Fig. 1B). As a control, the cell wall protein M49, represented by the monomer at 37 to 40 kDa (as predicted from the genome sequence) and the typical range of slower-migrating bands containing cell wall fragments (15), was found exclusively in the cell wall fraction, as expected (Fig. 1C). Therefore, Shr appears to remain associated with the cell membrane, probably by the transmembrane domain in its carboxy terminus.

The cellular location of Shr was further studied in an shr deletion-insertion mutant (ZE4912) (Fig. 2A) and the mutant strain complemented with shr expressed from its own promoter in the chromosome (ZE4924) (see Materials and Methods). To examine the effects of the shr mutation and of its complementation on the expression from the sia operon, we performed reverse transcription-PCR and Western blot analysis on RNA and proteins extracted from isogenic strains NZ131, ZE4912, and ZE4924. As can be seen in Fig. 2, the transcription of the siaA gene (Fig. 2B) and the production of this protein (Fig. 2C) were not significantly altered in either the mutant or the complementation strain in comparison to the parent strain. Therefore, the mutation in shr does not seem to be polar on the downstream genes in the sia operon. On the other hand, cell fractionation and Western blot analyses showed that the shr mutation in ZE4912 resulted in the loss of Shr from the membrane fraction (Fig. 2D, lane 2) and in the formation of a truncated Shr fragment (25-kDa) that was secreted into the culture supernatant (data not shown). The complementation of shr in ZE4924 strain restored the presence of the Shr protein in the cell membrane fraction (Fig. 2D, lane 3). These observations are consistent with the suggestion that the carboxy terminus of Shr is required for the Shr association with the cell membrane and with our previous observation that Shr has a leader peptide that can target its secretion.

To test whether membrane-bound Shr is exposed on the cell exterior or is buried within the peptidoglycan layer, we conducted whole-cell ELISA using Shr antibodies. GAS cells grown to stationary phase were used to coat ELISA plates and reacted with anti-Shr serum. An antiserum against the repressor protein MtsR (5) was used as a control for intracellular proteins, and normal rabbit serum (NRS) was used as a control
for any nonspecific reaction. As shown in Fig. 3, the Shr antiserum (black bars) reacted with the wild type (NZ131) and with the complementation strain (ZE4924). The specificity of the antiserum for Shr was demonstrated by its lack of reaction with the complementation strain (ZE4924). The specificity of serum (black bars) reacted with the wild type (NZ131) and representatives the average of at least three repeats, and the standard deviation (SD) is represented by the error bars. The significance of the difference in the bindings of anti-Shr serum to the three strains was examined by ANOVA (P = 0.001), followed by the Tukey HSD test for each strain pair. The asterisk indicates that the difference between the strains is significant (P < 0.01 for each strain pair).

**Shr is an MSCRAMM.** The surface localization and exposure of Shr raised the possibility that it may function in bacterial adherence in addition to iron uptake. We therefore examined the interactions of Shr with ECM components using ELISA assays. Different ligands immobilized onto microtiter plates were reacted with increasing concentrations of purified Shr. Shr binding was detected using Shr antiserum (Fig. 4). As a control for nonspecific interactions, wells coated with transferrin or goat IgG were incubated with the Shr antiserum directly. Only low background binding (OD405 of 0.1 ± 0.01) (Fig. 4 and data not shown) of the Shr antiserum to the control wells was observed, demonstrating the specificity of the Shr antibodies. Although Shr did not bind to transferrin (Fig. 4, diamonds) and goat IgG (data not shown), Shr bound to immobilized fibronectin (Fig. 4, triangles) and laminin (Fig. 4, squares) in a dose-dependent and saturable manner.

Inhibition experiments demonstrated the specificity of binding of Shr to the ECM ligands fibronectin and laminin. When Shr was allowed to interact with immobilized fibronectin in the presence of increasing concentrations of soluble fibronectin, the binding of Shr to the plates was inhibited in a dose-dependent manner (Fig. 5A, triangles). On the other hand, transferrin, which was not recognized by Shr in vitro, could not compete with immobilized fibronectin for binding to Shr (Fig. 5A, diamonds). Similarly, soluble laminin inhibited the binding of Shr to wells coated with laminin (Fig. 5B). Laminin also competed with fibronectin for Shr binding (up to 40% inhibition) (Fig. 5A, squares). However, at similar molar concentrations, laminin was less effective at competition than was soluble fibronectin (80% inhibition). This demonstrates that the binding of Shr is specific to a subset of ECM components.

GAS expresses multiple fibronectin binding proteins on its surface (22, 50). To test the ability of Shr to mediate bacterial binding to fibronectin in the absence of the other GAS fibronectin-binding proteins, we expressed Shr in a heterologous host. Plasmid pXL14, which carries the native shr gene, was introduced into *L. lactis* strain MG1363. The production of the Shr protein in *Lactococcus* was confirmed by Western blot analysis (data not shown), and the presentation of Shr on the cell surface was tested by ELISA. Microtiter plates were coated with cells of native *L. lactis* strain MG1363 or the recombinant strain that harbors pXL14 (MG1363/pXL14). The coated wells were reacted with the Shr antiserum (Fig. 6A, black bars), *L. lactis* antibodies (Fig. 6A, gray bars), or NRS (Fig. 6A, white bars). The Shr antiserum reacted significantly (P = 0.0037) only with bacteria that harbor the shr plasmid (pXL14), demonstrating specificity for Shr. As expected, the *L. lactis* antibodies interacted strongly and similarly with both strains MG1363 and MG1363/pXL14, and only weak binding of NRS to both strains was observed. Therefore, like in its native host, the Shr protein produced in *L. lactis* is exposed on the bacterial cell surface.

The binding of recombinant *L. lactis* cells expressing Shr to fibronectin and to transferrin was investigated. The two ligands...
were immobilized onto microtiter plates, and the coated wells were incubated with bacteria. The binding of *Lactococcus* to the immobilized ligands was detected using the *L. lactis* antiserum. Only weak binding of strain MG1363 to fibronectin (Fig. 6B, black bars), transferrin (Fig. 6B, white bars), or uncoated wells (data not shown) was observed, demonstrating that *L. lactis* does not interact strongly with fibronectin or transferrin. In contrast, MG1363 cells harboring plasmid pXL14 demonstrated significant (*P* < 0.001) binding to immobilized fibronectin (Fig. 6B, black bars); these cells, however, did not bind to transferrin (Fig. 6B, white bars). Therefore, *L. lactis* cells expressing Shr gained the ability to specifically bind to fibronectin. This observation demonstrates that Shr can mediate cell binding to the ECM and supports its possible role as a bacterial adhesin.

To test the function of Shr in the adherence of whole GAS cells directly, the *shr* mutant (ZE4912) was compared to its wild-type parent (NZ131) as well as to the mutant complemented with the *shr* gene (ZE4924). The binding of GAS to HEp-2 cells was assayed following incubation at 37°C for 2 h. In comparison to the wild-type strain, the *shr* mutant demonstrated about a 40% reduction in adherence (Fig. 7). This difference is statistically significant (*P* < 0.05). Although the complemented strain bound to the HEp-2 cells better than the mutant, it bound only about 75% as well as the wild type. The difference in binding between the complemented strain and either the wild type or the *shr* mutant was not statistically significant. Thus, although complementation was not complete, Shr seems to act as an adhesin in GAS attachment to HEp-2 cells.

Shr production and surface presentation are conserved among clinical isolates. A collection of 17 clinical isolates representing seven different M serotypes was examined for Shr expression using whole-cell ELISA. As summarized in Table 1, Shr antiserum reacted with most of the isolates (14 out of 17) and produced a signal that was at least twofold higher than the
background signal produced with the control serum NRS. However, a significant variation in the strength of the produced signal was found among the different clinical isolates. This range of reactions may result from differences in the amount of Shr protein produced by each strain or may be due to antigenic differences in Shr and subsequently weaker interactions with the Shr antiserum. The Shr sequence is very conserved among GAS genomes available in the database; however, the sequence variability may increase among clinical isolates. In summary, the Shr protein was present on the surface in 17 out of the 20 strains tested in this study (including NZ131, MGAS5005, and JRS4), suggesting that this protein is important for the GAS infection process.

Shr is expressed by GAS in vivo and elicits an immune response in mice. In cells growing in vitro in the laboratory, shr expression is regulated by the availability of iron in the medium (5). To determine if the conditions in vivo during GAS infection in a murine model allow the expression of Shr, we asked if a challenge with GAS leads to the production of Shr antibodies in mice. Mice were injected subcutaneously with strain MGAS5005 (M type 1), and sera collected 28 days after inoculation were assayed by ELISA for Shr antibody titers. Purified Shr was used to coat microtiter plates, which were then reacted with mouse sera (Fig. 8). Sera from mice injected with GAS reacted strongly with purified Shr in most of the animals tested (9 out of 14), whereas none of the sera obtained from naïve mice were reactive against Shr. The conversion of most mice to Shr positive demonstrates that Shr is immunogenic and that in most of the mice, it was produced during infection in sufficient amounts and durations to elicit an immune response.

Shr is important for GAS pathogenesis in the zebrafish infection model. The use of zebrafish as an infection model for pathogenic streptococci was previously established. i.m. injection of zebrafish with the native fish pathogen *Streptococcus iniae* causes a systemic disease, while injection with GAS causes mostly local muscle lesions and necrosis (34). The effect of shr inactivation on virulence was investigated via the i.m. infection route, as previously described (33, 34). Groups of six zebrafish were challenged with 10^6 CFU per fish of cells of wild-type strain NZ131, the shr mutant (ZE4912), or the mutant complemented with shr (ZE4924) (Fig. 9). Injection with the wild-type strain resulted in rapid fish death, with fish survival beginning to decline as early as 12 h postinfection, reaching a final survival rate of 22% at 48 h postinfection (Fig. 9, filled squares). On the other hand, the shr mutant strain was attenuated for virulence; fish injected with the mutant strain (Fig. 9, open squares) exhibited a slower decline in survival, and the final survival rate was significantly higher than that of fish infected with the wild-type strain (62% survival at 48 h postinfection; P < 0.0012). The complemented strain (Fig. 9, filled triangles) demonstrated intermediate virulence; fish infected with this strain died at a lower rate than that of fish infected with the wild-type strain (100% survival at 12 h and 24 h postinfection). However, the final survival rate eventually declined to a level similar to that of fish infected with the wild-type strain (25% survival at 48 h postinfection). Determination of LD_{50} values was done by injecting each fish with a range of 10^4 to 10^7 CFU of each strain. Consistent with the attenuated phenotype observed for the shr mutant, the wild-type LD_{50} value was significantly lower (5 × 10^4 CFU per fish)
than that of the mutant \((2.5 \times 10^6 \text{ CFU per fish})\), and an intermediate \(L D_{50}\) value \((5.6 \times 10^5 \text{ CFU})\) was observed with the complemented strain, demonstrating a partial complementation of virulence.

The impact of Shr on the infection process in GAS was also evaluated by testing the ability of the \(shr\) mutant to compete with the wild-type strain for growth in the muscle tissue during coinfection. Zebrafish were inoculated by i.m. injection with \(10^6\) CFU of a mixed culture comprised of a 1:1 ratio of wild-type to mutant cells. The lesion area was excised 24 h postinfection, and the number of viable bacteria of each strain in the tissue was determined by comparing the bacterial recovery on plates with spectinomycin (the mutant’s marker) to that on antibiotic-free plates. The CI is defined as the output ratio of mutant to wild-type bacteria divided by the input ratio of mutant to wild-type bacteria. The CI of the \(shr\) mutant was calculated to be \(0.011 \pm 0.002\). The growth rate of the \(shr\) mutant in vitro in THYB was identical to that of the wild-type strain. In addition, the chromosomal mutation in \(shr\) was stable during growth in THYB in the absence of spectinomycin, as was evident from the plating efficiency (100%) of this strain on selective plates after overnight growth in the absence of antibiotics. Therefore, the low CI suggests that the inactivation of \(shr\) dramatically reduced the in vivo fitness of GAS in comparison to the wild-type strain in zebrafish tissue. Since Shr is found both on the cell surface and in the culture supernatant, the fact that the presence of the wild-type strain during infection could not rescue the attenuated phenotype of the mutant suggests that the surface-associated form of Shr is important for GAS fitness during infection.

**DISCUSSION**

The human pathogen GAS exports numerous proteins to its surface, where they carry out the many tasks that mediate colonization and disease. Examples of these tasks include the escape and subversion of the immune response, adherence, and nutrient scavenging (6). Shr is a large, complex, and highly conserved surface protein in GAS (Fig. 1A) that does not share extensive sequence homology with other proteins found in the database (4). Although the functional role of Shr has not yet been deciphered, this protein has been shown to bind hemoproteins and is also suggested to mediate heme acquisition and transport in conjunction with Shp and the SiaABC heme transporter (4, 36). The major objectives of this study were to explore the function of Shr and its role in GAS pathogenesis in depth. The results show that the contribution of Shr to the GAS infection process extends beyond iron uptake to include interactions with the ECM and adherence to epithelial cells. Therefore, Shr represents a new MSCRAMM in GAS that is necessary for virulence in the zebrafish model of necrotizing fasciitis.

When GAS is grown in culture under laboratory conditions, Shr is found in the supernatant as well as in association with whole GAS cells. Even though Shr contains a leader peptide that can mark proteins for secretion (4), the mechanism that facilitates the association of Shr with the GAS surface remains unclear. The majority of surface proteins in gram-positive bacteria are covalently attached to the peptidoglycan by a sortase enzyme, which recognizes a C-terminal cell wall-sorting motif followed by a hydrophobic segment and a charged tail (31, 45). The LPXTG sequence serves as the sorting signal for the housekeeping sortase in GAS. Shr does not have an LPXTG motif in its C terminus or the QVPTG signal that is recognized by \(srtC2\), an accessory sortase that is also produced by GAS strain NZ131 (3). Using cell fractionation and immunoblotting, this study demonstrated that Shr is not bound to the cell wall; rather, Shr is found in the membrane fraction (Fig. 1B). The absence of recognizable cell wall sorting signals in the Shr sequence is consistent with these findings. It seems likely that Shr remains associated with the cytoplasmic membrane after its export to the surface due to the putative transmembrane segment and charged tail found in its C terminus (Fig. 1A), as was found to be the case for ActA of *Listeria monocytogenes* (21). Shr is not present in the membrane of mutant GAS strain ZE4912, since the mutation is located in the \(5'\) coding region of the \(shr\) gene and therefore results in the formation of a truncated protein secreted into the culture supernatant (Fig. 2 and data not shown). The secretion of the abridged Shr fragment supports the hypothesis that the C terminus in Shr is required for its membrane anchoring and therefore for its association with GAS cells.

The ability of anti-Shr serum to recognize Shr on the surface of whole GAS cells (strains NZ131 and ZE4924) (Fig. 3, black bars) shows that the wild-type Shr protein spans the cell wall in GAS and is exposed to the extracellular environment. Therefore, Shr may be able to interact with large extracellular molecules, in addition to small ligands capable of diffusing the cell wall. Since Shr appears to be anchored to the cytoplasmic membrane through its C terminus, it is possible that it can deliver heme from the extracellular compartment directly to the transport components found in the cell membrane. This heme transport scheme is different from the one proposed previously by Maresso and Schneewind for heme uptake by the Isd proteins in *Staphylococcus aureus*, where heme is relayed in a cascade fashion from surface-exposed NEAT proteins, such as IsdA, IsdB, or IsdH, to an IsdC protein that is found deeper in the peptidoglycan; heme is then transferred by IsdC to the IsdDEF membrane transporter for uptake (30).

Using solid-phase binding assays, we were able to demonstrate that Shr also binds the ECM components fibronectin and laminin (Fig. 4). Competition studies performed with soluble and immobilized fibronectin and laminin demonstrated that Shr interactions with these adhesive molecules are specific (Fig. 5). Therefore, Shr appears to have a broader spectrum of ligands than previously suggested. Shr availability on the GAS surface combined with its affinity for ECM components qualifies this receptor as a new MSCRAMM in GAS. The report also states that recombinant *L. lactis* cells that express Shr on the surface bind specifically to immobilized fibronectin (MG1363/pXL14) (Fig. 6), which is an activity not found in native *L. lactis* cells (MG1363) (Fig. 6). This observation shows that Shr is able to mediate bacterial attachment to the ECM and further supports its role as an MSCRAMM. Interestingly, the Shr amino acid sequence does not contain the typical fibronectin-binding repeats found in other fibronectin MSCRAMMs (22, 44). Thus, it is not clear which part of this large protein mediates the binding to fibronectin or to laminin. It was previously reported that the NEAT protein IsdA in *S. aureus* binds to several nonheme host proteins,
including the ferric carrier transferrin (51), several matrix and plasma proteins, and hemoglobin (8). The results presented here demonstrate that Shr does not bind to transferrin, as it has a binding pattern similar but not identical to that of IsdA. The ligand binding in IsdA is attributed to its single NEAT domain, but the residues involved in binding to the serum and ECM proteins were not determined. The sequence homology between IsdA and Shr is limited to the NEAT regions and is quite low even within these domains. Moreover, Shr is a significantly larger and more complex protein than IsdA (145 and 38 kDa, respectively) and has a central leucine-rich repeat (LRR) segment, a unique amino-terminal region, and two NEAT domains. Additional analysis is required to determine the domains and the residues involved in the recognition of various Shr ligands.

The data presented show that Shr helps facilitate GAS attachment to HEP-2 epithelial cells, where a reduction in binding of about 40% was observed in the shr mutant. This decrease in adherence is statistically significant and can be partially restored in the complementation strain (Fig. 7). Since GAS expresses several adhesins that mediate binding to HEP-2 cells, it is not surprising that only a small reduction in adherence was observed in the shr mutant. As far as we can determine, Shr is the only adhesin reported to be induced in response to the iron restriction likely encountered during infection. Therefore, Shr’s contribution to adherence may be more significant under such disease-specific conditions. Shr-dependent adherence may take place through fibronectin binding or additional serum-bridging molecules, as found for other GAS adhesins (22, 50). Alternatively, Shr may interact directly with a host cell receptor via a mechanism that may be assisted by its LRR domain (Fig. 1A). The LRR was previously suggested to provide a scaffold for the formation of protein-protein interactions (20), and the LRR domain in internalin was shown to be necessary and sufficient for binding to E-cadherin in L. monocytogenes (25).

It seems likely that shr complementation was not able to restore adherence to the level observed in the wild type due to the nature of the mutation and the method used for complementation. The shr mutation in ZE4912 results in the production of a truncated Shr fragment that is secreted into the extracellular medium. This Shr fragment, which is also produced by the complementation strain, may serve as competitor interfering with Shr-mediated attachment to epithelial cells and impairing adherence in the complementation strain. In addition, complementation strain ZE4924 is a merodiploid strain created by Campbell insertion. Since this type of mutation is unstable, it may excise and reduce the efficiency of complementation in the binding assays.

This study presents several experiments that suggest that Shr is important for GAS virulence. A single infection event was found to be sufficient to trigger a significant antibody response to Shr in convalescent mice, indicating that Shr is expressed in vivo in an adequate amount and an adequate duration to elicit a host response (Fig. 8). While the majority of the injected mice (9 out of 14) developed high Shr titers, the anti-Shr antibody levels in the remaining five mice were low. The observed variations in the antibody response following GAS challenge may result from differences in the time that it took individual mice to clear the bacteria, from variations in the efficiency of their immune response, or from both. Analysis of Shr production in different GAS strains, including a collection of 17 clinical isolates (Table 1), revealed that Shr could be detected on the surface of most of the examined strains. The inactivation of shr resulted in an attenuation of virulence in a zebrafish model (ZE4912) (Fig. 9), with the LD₅₀ of the mutant being about 50 times higher than that of the wild-type strain. As in the adherence assay, only a partial restoration of virulence was observed with the complemented strain. Both the kinetics of survival in zebrafish postinfection and the LD₅₀ value of complementation strain ZE4924 demonstrated an intermediate level of virulence in comparison to those of wild-type strain NZ131 and shr mutant strain ZE4712. This suggests that either an instability of the complemented strain or the production of both full-length and a truncated fragment of Shr by ZE4924 prevented a complete recovery of virulence. The recovery of the mutant strain from the lesion tissue was much less than that of the wild-type strain after coinfection (CI of 0.01). This observation demonstrates that the inactivation of shr results in a mutant strain that is significantly less fit than the wild-type parent strain. Shr is found both in the culture supernatant and on the cell surface. The inability of the wild-type strain to complement the in vivo growth defect of the mutant strain during coinfection of zebrafish muscle strongly suggests that it is the surface-anchored form of Shr that is important for the ability of GAS to persist in the host during infection.

In summary, given that Shr is a broad-spectrum surface receptor contributing to iron acquisition (4; data not shown), ECM binding, and adherence, its contribution to the disease process in GAS appears complex. Ongoing research is under way to further investigate this important multifunctional surface protein and to better understand its role in the disease process.

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REFERENCES


16. Lbp, is involved in


