Impact of the SpeB Protease on Binding of the Complement Regulatory Proteins Factor H and Factor H-Like Protein 1 by *Streptococcus pyogenes*

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Microbial pathogens often exploit human complement regulatory proteins such as factor H (FH) and factor H-like protein 1 (FHL-1) for immune evasion. Fba is an FH and FHL-1 binding protein expressed on the surface of the human pathogenic bacterium *Streptococcus pyogenes*, a common agent of pharyngeal, skin, and soft-tissue infections. Fba has been shown to contribute to phagocytosis resistance, intracellular invasion, and virulence in mice. Here, we look at the role of Fba in recruitment of FH and FHL-1 by five serotype M1 isolates of streptococci. Inactivation of *fba* greatly inhibited binding of FH and FHL-1 by all isolates, indicating that Fba is a major FH and FHL-1 binding factor of serotype M1 streptococci. For three isolates, FH binding was significantly reduced in stationary-phase cultures and correlated with high levels of protease activity and SpeB (an extracellular cysteine protease) protein in culture supernatants. Analysis of a *speB* mutant confirmed that SpeB accounts for the loss of Fba from the cell surface, suggesting that the protease may modulate FH and FHL-1 recruitment during infection. Comparisons of *fba* DNA sequences revealed that the FH and FHL-1 binding site in Fba is conserved among the M1 isolates. Although the ligand binding site is not strictly conserved in Fba from a serotype M49 isolate, the M49 Fba protein was found to bind both FH and FHL-1. Collectively, these data indicate that binding of FH and FHL-1 is a conserved function of Fba while modulation of Fba function by SpeB is variable.

Many infectious agents, including viruses, fungi, and bacteria, utilize host complement regulatory proteins for immune evasion and host colonization (60). Two fluid-phase regulators of complement activation commonly recruited by pathogens are factor H (FH) and factor H-like protein 1 (FHL-1), two proteins encoded by the same gene (22, 27, 83). FH is a 150-kDa protein composed of 20 repeat elements known as short consensus repeats (SCRs). Each SCR constitutes an independently folded domain of approximately 60 amino acid residues. FHL-1 is a 42-kDa protein composed of seven SCRs that are identical to SCRs 1 to 7 of FH with four additional amino acids at the C terminus (83). The ability of bacterial pathogens to evade complement attack and opsonophagocytosis is often influenced or dictated by a pathogen's ability to bind FH or other complement regulatory proteins (1, 19, 20, 39, 40, 42, 47, 73). Recruitment of FH or FHL-1 can facilitate both dissociation of C3 convertase and cleavage of cell surface-bound C3b to iC3b. Because iC3b does not participate in C3bBb formation, C3b cleavage can result in decreased deposition of C3 fragments on cell surfaces (17, 28, 36, 38, 59, 60).

The gram-positive bacterium *Streptococcus pyogenes*, or group A streptococcus (GAS), is a major human pathogen causing infections ranging from superficial infections of the throat (pharyngitis) or skin (impetigo) to highly invasive and potentially lethal infections such as necrotizing fasciitis and strepto-

coccal toxic shock syndrome (16). Horstmann et al. (36) first proposed that the acquisition of FH by GAS contributes to the bacterium's capacity to evade phagocytosis by polymorphonuclear leukocytes. For many GAS isolates, FH binding is mediated by M proteins, a family of cell surface, antiphagocytic proteins (25, 26). Some types of M proteins have also been shown to bind FHL-1 (42, 46). It should be noted, however, that GAS resistance to phagocytosis is a complex phenotype, resulting from the collective activities of numerous virulence factors, including capsules, secreted proteins, and cell surface proteins (4, 13, 18, 34, 49, 68, 81, 82). Because of this, recruitment of complement regulatory factors, or even expression of M protein, is not essential for some GAS isolates to resist phagocytosis (41–43, 45, 64, 68).

We recently identified a novel protein expressed by a serotype M1 GAS isolate, 90-226, that mediates binding of both FH and FHL-1 (61). The protein, Fba, is the first non-M-like protein of GAS shown to bind these complement regulatory factors. We previously reported that Fba contributes to the phagocytosis resistance of GAS (61). Additionally, we demonstrated that Fba, via binding of FHL-1, can promote the entry of GAS into human epithelial cells (62). The *fba* gene is present in GAS of numerous serotypes, suggesting that the protein contributes to GAS survival in its host (70, 78). In fact, Terao et al. (78) reported that Fba contributes to GAS virulence in mice. The N- and C-terminal regions of Fba are well conserved among strains with various alleles, but the amino acids in the central region of the protein can differ between strains (70, 78). It is unknown to what extent these differences in amino acid sequence affect the functions of the protein; however, an earlier study suggested that the Fba protein of a serotype M49 GAS isolate does not bind FH (70).

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Description	Reference or source
Strains		
<i>E. coli</i>		
XL-1 Blue	<i>recA1</i>	Stratagene
BL21	<i>ompT</i>	Amersham Biosciences
<i>S. pyogenes</i>		
90-226	M1 ⁺ Fba ⁺	14
DC283	<i>fba</i> derivative of 90-226	62
DC349	<i>speB</i> derivative of strain 90-226	This study
80-003	M1 ⁺ Fba ⁺	11
87-263	M1 ⁺ Fba ⁺	11
AP1	M1 ⁺ Fba ⁺	12
CS101	M49 ⁺ Fba ⁺	41
MGAS5005	M1 ⁺ Fba ⁺	35
Plasmids		
pFW5	GAS suicide vector	71
pFW5Δ <i>fba</i>	730-bp internal fragment of <i>fba</i> cloned into pFW5	61
pLW100	691-bp internal fragment of <i>speB</i> cloned into pFW5	This study
pGEX-2T	Plasmid expression vector	Amersham Biosciences
pVP138	Fba ₉₀₋₂₂₆ 37-324 expression plasmid	62
pVP155	Fba ₉₀₋₂₂₆ Δ68-104 expression plasmid	62
pMV3	Fba ₉₀₋₂₂₆ 69-112 expression plasmid	This study
pVP163	Fba _{CS101} 38-348 expression plasmid	This study
pVP172	Fba _{CS101} Δ71-125 expression plasmid	This study
pMV1	Fba _{CS101} 71-125 expression plasmid	This study
pYT1143	GAS shuttle vector carrying <i>fba</i>	78
pBlueBac4.5/V5-His	Vector for crossing FHL-1 open reading frame into baculovirus	Invitrogen
I.M.A.G.E. clone 267249	FHL-1 cDNA clone	American Type Culture Collection (77)
pGEM-T Easy	Cloning vector	Promega Corporation

Deletion analysis of Fba from GAS strain 90-226 localized the major binding site for FH and FHL-1 to a putative coiled-coil-forming region in the N-terminal half of the protein. Pep-Spot analyses confirmed that a linear peptide sequence that overlaps the coiled-coil region (YKQIKTAPDKDKLLF) directly interacts with FH and FHL-1 (62). Interestingly, this sequence is not strictly conserved in all Fba proteins, suggesting that some proteins may not bind FH or FHL-1 or may bind with a different affinity than that of the protein from strain 90-226 (70, 78). The Fba binding site in FH and FHL-1 has been localized to SCR 7, a domain common to both proteins (83). SCR 7 also contains binding sites for heparin and M proteins. Accordingly, heparin can inhibit binding of FH by Fba or M proteins (46, 62, 76, 83).

Although Fba binds both FH and FHL-1, strain 90-226 selectively binds FHL-1, rather than FH, when incubated with human plasma or serum (61). This result was surprising as the concentration of FH in human plasma (400 μg/ml) is significantly higher than that of FHL-1 (10 to 50 μg/ml) (32). An *fba* mutant of strain 90-226 does not bind appreciable amounts of either ligand from plasma (61). Selective binding of FHL-1 has also been observed for other GAS isolates, but the GAS factor(s) that mediates binding was not identified (43, 63).

Fba possesses a conserved amino acid sequence (LPSTG) near the C terminus to anchor the protein to the bacterial cell wall (25). Several lines of experimentation have confirmed that Fba is produced and anchored to the cell surface during the exponential phase of growth (61, 78). In our studies with a single GAS strain (strain 90-226), however, we observed that Fba was not present on the surface of stationary-phase streptococci. GAS grown to stationary phase in media containing

E64, a cysteine protease inhibitor, retained Fba on the cell surface (61). While these results suggested that the GAS SpeB protease, an extracellular cysteine protease expressed in the late exponential to early stationary phases of growth (8), was responsible for the loss of Fba from stationary-phase cells, this possibility had not been directly tested. Investigation of a possible role for SpeB in the loss of Fba was warranted, as many GAS isolates do not produce significant amounts of SpeB during growth in vitro (8, 12, 54). Moreover, culture supernatants of some GAS isolates, such as strain 90-226, often contain temperate bacteriophage, suggesting that bacterial cells are subject to lysis during normal growth, a process that could release intracellular proteinases into the culture medium. Thus, it was plausible that strain 90-226 expresses a proteinase other than SpeB that affects cell surface presentation of Fba.

Here we extend our previous studies by investigating the roles of Fba and SpeB in the recruitment of FH and FHL-1 by other serotype M1 isolates of GAS. Our results demonstrate that Fba is a major FHL-1 and FH binding factor of this serotype that selectively binds FHL-1 from human plasma. We also confirmed that SpeB is involved in the loss of Fba from stationary-phase streptococci. Perhaps more importantly, we determined that strain 90-226 is neither a unique nor a representative strain with regard to stationary-phase expression of Fba. Finally, we established that the Fba protein of a serotype M49 strain, CS101, can bind both FH and FHL-1, suggesting that this is a conserved function of Fba.

MATERIALS AND METHODS

Bacterial strains and culture media. The bacterial strains used in this study are listed in Table 1. Streptococci were grown in Todd-Hewitt broth supplemented

TABLE 2. Oligonucleotides used in this study

Oligo-nucleotide	Sequence ^a
aad9-2	CTGCTAACAAATTTCTCTCC
fba F3	GTTGGATCCGCGTAGATGGCATCCCTCAAT
fba F6	GTTGGATCCCGAGTTGAGGCACCCGTGCAG
fba F9	GCTGGATCCTTAGGTATAAGCTTAGAAGCTGCC AAAGAG
fba R3	AGTGAATTTCTCACTGTTGAAGGCAAGTACTTA CCTG
fba R7	AGTGAATTTCTCAGAATAATAGCTTATCTTTGTC TGGTGCAGT
fba CF1	GTTGGATCCGCTAGATAACGGGCGACAAATCTC TTTGACTC
fba CF2	GCTACCGGTCCAGCGTCTACTGAGCCTACTAC
fba CF3	GCTGGATCCTTAGGTATAAGCTTAGAAGCTGCC AAAG
fba CR2	AGTGAATTCCTTACCTGACTCATAGCCCCCT AGCC
fba CR3	CGGGATGAATTTCTCAAAGATTCTTAATATCTTT AGAGTACTCACT
fba CR4	GCTACCGGTACCTAAAGGAATAAGGCCATCC
FHL-1 F1	AATGGATCCGGTTATGGCAAGACTTCTAGCAA AGATTATTTGCTTATG
FHL-1 R1	ATAACCGGTTCCGCCTCCGAGGGTAAAGCTGA CACGGATGC
speB F1	ATCGGATCCTGCACGAAGCGCAGAAGATAT
speB F3	GGTTTGATGCCTACAACAGC
speB R1	ATCGGATCCAGCTACCTGCAGAACCCTA
Spy2009 F1	TTAAGGATCCAGGAGGACAATATGCGTAGAGC

^a Relevant restriction endonuclease cleavage sites are underlined.

with 1% yeast extract (THY broth; Difco Laboratories, Detroit, Mich.). Solid media for streptococci were Todd-Hewitt or sheep blood agar. *Escherichia coli* was grown in Luria-Bertani broth. Solid medium contained 1.5% agar. When appropriate, the following antibiotics were added to bacterial culture media at the indicated concentrations: spectinomycin, 100 µg/ml for GAS and *E. coli*; erythromycin, 1 µg/ml for GAS and 350 µg/ml for *E. coli*; and ampicillin, 100 µg/ml for *E. coli*. In some experiments, streptococci were cultured in THY medium containing a 200 µM concentration of the cysteine protease inhibitor E64 (Sigma-Aldrich). Plasmid transformations of GAS were performed as described previously (6).

General DNA techniques. Isolations of plasmid DNA from *E. coli* and genomic DNA from GAS were performed using reagents purchased from Promega Corp., Madison, Wis. DNA sequencing was performed by the Kansas University Medical Center Biotechnology Support Facility. PCR was performed using standard procedures (75). The oligonucleotide primers used in this work are listed in Table 2. PCR for the detection of *fba* in GAS isolates was performed with the oligonucleotide primers Fba F3 and Fba R3.

Cloning and sequencing of *fba* alleles. Genomic DNA was isolated from GAS strains 80-003, 87-263, AP1, and MGAS5005. The *fba* genes were amplified via PCR using oligonucleotides Spy2009 F1 and fba R3. The primers are homologous to sequences overlapping the *fba* start codon and a sequence coding for the LPXTG motif, respectively. The PCR fragments were gel purified and ligated with pGem-T Easy (Promega) by the use of reagents and a protocol obtained from the manufacturer. The sequences of the cloned *fba* genes were determined from the region encoding the amino-terminal signal sequence through the region encoding the LPXTG motif. The sequence of the *fba* gene of MGAS5005 is identical to that of strains 90-226 and SS-9 (78). The *fba* genes of 80-003 and 87-263 are identical to that of GAS strain SF370 (24). The *fba* gene of the AP1 strain is novel.

Insertional inactivation of *fba* and *speB*. The *fba* gene of each GAS isolate was inactivated by electroporation with pFW-5Δ*fba* (61) and selection for spectinomycin-resistant (Spc^r) transformants. This plasmid carries a 730-bp internal fragment of *fba* cloned into the suicide vector pFW5 (71). To verify integration of the plasmid into *fba*, genomic DNA was isolated from Spc^r transformants, and PCRs were performed with two pairs of oligonucleotide primers, i.e., primers fba F1 and fba R3 and primer fba R3 and the pFW5-specific oligonucleotide primer aad9-2.

For insertional inactivation of *speB*, a 69-bp internal fragment of the gene was amplified using the oligonucleotide primers speB F1 and speB R1. Genomic

DNA from strain 90-226 was used as the template. The amplicon was digested with BamHI and ligated with BamHI-restricted pFW5. The structure of the resulting plasmid was verified by DNA sequencing and used for transformation of strain 90-226. Integration of the plasmid into *speB* was verified via PCR using oligonucleotide primers speB F3 and aad9-2.

Subcloning of FHL-1 cDNA and construction of FHL-1-Bac. A plasmid containing the complete cDNA for human FHL-1 (I.M.A.G.E. clone 5267249) was obtained from the American Type Culture Collection (77). The coding region, including the native signal sequence, was amplified via PCR using oligonucleotide primers FHL-1 F1 and FHL-1 R1 (Table 2). The amplicon was digested with BamHI and AgeI and ligated with BamHI-AgeI-digested pBlueBac4.5/V5-His (Invitrogen). The structure of the resulting plasmid, pFHL-1, was verified by DNA sequencing. *Spodoptera frugiperda* (Sf9) cells (Invitrogen) were then co-transfected with pFHL-1 and linearized DNA of the baculovirus vector Bac-N-Blue (Invitrogen). Homologous recombination between pFHL-1 and the baculovirus resulted in recombinant baculovirus carrying the FHL-1 open reading frame. The recombinant virus (FHL-1-Bac) was plaque purified and amplified by three rounds of replication in Sf9 cells (37). PCR analysis confirmed the presence of the FHL-1 coding region in FHL-1-Bac. Western blots were performed to verify expression of recombinant FHL-1 by infected Sf9 cells.

Construction of plasmids for expression of recombinant Fba proteins. Plasmid pVP163 was constructed for expression of the mature form of Fba_{CS101} (amino acid residues 38 to 348) as a recombinant protein (70). Oligonucleotide primers fba CF1 and fba CR1 were used to amplify the *fba* gene of GAS strain CS101. The amplicon was digested with BamHI and EcoRI and ligated with restricted pGEX-2T. To construct pVP172, the oligonucleotide primers fba CF1 and fba CR4 were used to amplify the region of *fba*_{CS101} coding for amino acid residues 38 to 70. The PCR product was digested with BamHI and AseI. The region coding for amino acid residues 126 to 348 of Fba_{CS101} was amplified using the fba CF2 and fba CR1 oligonucleotide primers. The PCR product was digested with AseI and EcoRI. The two restricted, gel-purified DNA fragments were then ligated with BamHI-EcoRI-digested pGEX-2T. The protein encoded by pVP172 (Fba_{CS101}Δ71-125) is Fba_{CS101} with amino acid residues 71 to 125 deleted. Plasmid pMV2 was constructed for expression of the amino acid residues 69 to 125 of Fba_{CS101} as a glutathione S-transferase (GST) fusion protein. A portion of Fba_{CS101} was amplified using oligonucleotide primers fba CF3 and fba CR3, and the PCR product was digested with BamHI and EcoRI, gel purified, and ligated with pGEX-2T. An analogous construct for expression of amino acid residues 69 to 112 of Fba₉₀₋₂₂₆, pMV1, was made using oligonucleotide primers fba F9 and fba R7 in the initial PCR.

Proteins, antibodies, and sera. Human FH was obtained from Quidel Corp., Santa Clara, Calif. Recombinant FHL-1 protein was purified from culture supernatants of FHL-1-Bac-infected High Five (*Trichoplusia ni*) cells (Invitrogen) as described previously (48). Recombinant Fba proteins were purified from cultures of *E. coli* BL21 carrying derivatives of pGEX-2T (pVP138, pVP155, pVP163, and pVP172) as previously described (62). SpeB was purified from culture supernatants of GAS strain 90-226 as previously described (21). The GST tag was removed from these proteins by thrombin cleavage (Amersham Biosciences). Fba_{CS101} 71-125, Fba₉₀₋₂₂₆ 69-112, and GST were purified in a similar manner except that thrombin cleavage was not performed. Goat anti-human FH was purchased from Quidel Corporation. Donkey anti-goat immunoglobulin G (IgG), labeled with horseradish peroxidase or alkaline phosphatase (AP), was from Chemicon International, Temecula, Calif. Mouse anti-rabbit IgG conjugated with AP was obtained from Sigma Chemical Company, St. Louis, Mo. Donkey anti-rabbit IgG conjugated with fluorescein isothiocyanate (FITC) was obtained from Jackson ImmunoResearch Laboratories, West Grove, Pa. Rabbit anti-Fba and preimmune sera were produced by Cocalico Biologicals Inc., Reamstown, Pa. (62). SpeB antiserum was purchased from Toxin Technology, Inc., Sarasota, Fla. Human plasma was obtained from healthy adult volunteers in accordance with a protocol approved by the Kansas University Medical Center Human Subjects Institutional Review Board. Protein mass standards were purchased from Bio-Rad Laboratories, Hercules, Calif.

Assays for binding of FH and FHL-1 by GAS and Fba proteins. Binding of FH and FHL-1 to immobilized GAS was assayed as previously described (15, 61). Unless stated otherwise, bacteria were harvested from log-phase cultures (optical density at 560 nm [OD₅₆₀], approximately 0.5) grown in THY medium. Bacterial cells were harvested by centrifugation, washed once with phosphate-buffered saline (PBS), and suspended to an OD₅₆₀ of 0.05 in 50 mM carbonate buffer, pH 9.6. Wells of microtiter plates were coated with suspensions of bacterial cells overnight at 5°C. For controls, some wells were mock coated with carbonate buffer. After removal of unbound cells, the wells were incubated with wash buffer (PBS containing 0.5% gelatin and 0.05% Tween 20) for 60 min at 37°C. Except where stated otherwise, 100 µl of wash buffer containing 10 µg of purified FH or

FHL-1/ml was then added to the wells, and the plates were incubated for 2 h at room temperature. For controls, buffer only was added to wells coated with each bacterial strain. After the wells were washed to remove unbound FH, 100 μ l of wash buffer containing FH antibody was added to the wells, and the plates were incubated for 60 min at 37°C. After unbound antibody was removed, wash buffer containing an AP-labeled secondary antibody was added, and the incubation was repeated. Finally, the wells were washed, and 200 μ l of 0.1 M glycine [pH 10.5] containing 1.5 mg of *p*-nitrophenylphosphate/ml, 1 mM CaCl_2 , and 1 mM ZnCl_2 was added to each well. The plates were incubated at 37°C, and absorbance values at 405 nm were determined. For each strain, absorbance values for wells coated with bacteria but not incubated with FH or FHL-1 were subtracted as background. The subtracted absorbance values typically ranged from 0.025 to 0.1. The subtracted values were slightly higher than absorbance values obtained for the mock (carbonate buffer)-coated wells incubated with FH or FHL-1. Data are from three independent experiments in which each strain was assayed three times. In separate experiments, each GAS strain was labeled with FITC (Sigma-Aldrich) (80) and applied to microtiter plates in order to verify that comparable numbers of streptococci bound to the plates. Under the conditions we used for adsorption, binding levels did not significantly differ between strains and were unaffected by mutations in *fba*. Assays of FH and FHL-1 binding to recombinant Fba proteins were performed similarly. For these experiments, microtiter wells were coated with 2 μ g of Fba protein/ml.

Plasma adsorption experiments. Plasma adsorption experiments were performed essentially as described previously (39, 61). GAS strains were grown to an OD_{560} of 0.5. Bacteria were isolated by centrifugation, washed twice with PBS containing 0.05% Tween 20 (PBST), and then suspended in PBST to approximately 10^{10} CFU/ml. One-hundred-microliter portions of the cell suspensions were mixed with 100 μ l of human plasma and incubated with gentle rocking at room temperature for 1 h. The mixtures were then centrifuged for 10 min at $2,000 \times g$ at room temperature. The resulting pellets were washed five times with 500 μ l of PBST containing 25 μ M E64 and 1 mM phenylmethylsulfonylfluoride (Sigma). The cell pellets were then suspended in 100 μ l of 0.1 M glycine, pH 2.0, and incubated at room temperature for 10 min. The bacteria were pelleted by centrifugation at $10,000 \times g$ and the resulting supernatants were transferred to new tubes. The supernatants were neutralized with NaOH and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Gels were either stained with Coomassie blue or transferred to nitrocellulose membranes. To detect FH and FHL-1, membranes were successively incubated with 20 mM Tris [pH 7.5]–0.5 M NaCl–0.05% Tween 20 (TBST) containing 0.5% gelatin, TBST containing 0.5% gelatin and FH antiserum, and donkey anti-goat IgG conjugated with AP. Blots were developed with nitroblue tetrazolium chloride and 5-bromo-4-chloro-3-indolylphosphate *p*-toluidine (Invitrogen).

Proteinase activity in culture supernatants and Western blots for SpeB. Proteinase activity in culture supernatants was assayed as previously described (3, 12). GAS were cultured for 14 h in THY broth, and the OD_{560} was measured for each culture. Bacteria were pelleted by centrifugation, and the resulting supernatants were filter sterilized. To assay proteinase activity, 200 μ l of supernatant, or 100 μ l of supernatant plus 100 μ l THY broth, was mixed with 200 μ l of activation buffer (0.1 M sodium acetate [pH 5.0], 20 mM dithiothreitol, 1 mM EDTA) and incubated at 37°C for 60 min. A 200- μ l volume of activation buffer containing 2% azocasein was then added to each sample, and the mixtures were incubated for 60 min at 37°C. One milliliter of 6% trichloroacetic acid was then added to each sample, and the mixtures were centrifuged at $15,000 \times g$ for 5 min. The OD_{366} was then determined for each supernatant, and the values corrected to compensate for the various ODs of the original bacterial cultures. Each sample was assayed four times in each of three independent experiments.

To detect SpeB in culture supernatants, ethanol was added to filter-sterilized supernatants to a final concentration of 75% (21). The mixtures were incubated overnight at -20°C and centrifuged at $20,000 \times g$ for 10 min, and the resulting pellets were suspended in approximately 1/10 volume of sterile H_2O . The amounts of H_2O used for suspension were adjusted to compensate for the various optical densities of the original bacterial cultures. Samples were fractionated on SDS–10% PAGE gels and transferred to a polyvinylidene difluoride membrane (Bio-Rad). Membranes were blocked with 5% skim milk–1% Tween 20 in PBS and then incubated successively with SpeB antiserum and donkey anti-rabbit IgG labeled with horseradish peroxidase. Supersignal chemoluminescent substrate (Pierce Chemical Co.) was added to the membranes, which were then exposed to XAR film (Kodak). As a control for nonspecific binding of antibodies, blots were subsequently stripped by incubation in 0.2 M glycine, pH 2.8, containing 0.1% Tween 20 and 3% bovine serum albumin, blocked, and then reprobed with preimmune rabbit serum. These experiments confirmed that the bands present in the SpeB blots did not react with preimmune serum. The

FIG. 1. Growth phase effects on binding of FH by GAS isolates. Wells of microtiter plates were coated with GAS grown to mid-exponential phase (hatched bars), stationary phase (gray bars), or stationary phase in the presence of the cysteine protease inhibitor E64 (open bars). The immobilized cells were incubated with FH followed by FH antiserum and a labeled secondary antibody. FH binding is expressed relative to the wild-type M1 isolate, strain 90-226. Data are from two independent experiments, in which each assay was performed three times. Error bars represent standard deviations.

exceptions were SpeB preparations from strain CS101, for which three bands ranging from 30 to 40 kDa reacted with both sera.

Extraction and analysis of cell surface proteins. GAS were isolated via centrifugation from stationary-phase cultures grown in either THY medium or THY medium supplemented with E64. Harvested cells were washed twice with 10 mM Tris [pH 8]–1 mM EDTA–25% sucrose and then suspended in 1/4 volume of 10 mM Tris [pH 8]–1 mM EDTA–25% sucrose containing 1 mg of lysozyme/ml, 500 U of mutanolysin/ml, 100 μ g of RNase A/ml, 25 μ M E64, and 1 mM phenylmethylsulfonylfluoride (61, 65). The suspensions were incubated at 37°C for 30 min and then centrifuged at $2,500 \times g$ for 10 min at 4°C. Trichloroacetic acid was added to the resulting supernatants to a final concentration of 16% (vol/vol), and the mixtures were incubated on ice for 20 min. The mixtures were then centrifuged at $11,500 \times g$ for 10 min at 4°C. The pellets were washed with acetone and suspended in 50 mM NaOH. The preparations were fractionated by SDS-PAGE and either stained with Coomassie blue or transferred to nitrocellulose. To detect Fba, the membranes were blocked with TBST containing 0.5% gelatin and then incubated with Fba antiserum and a labeled secondary antibody.

Immunofluorescence microscopy. Fba was detected on the surface of streptococci as previously described (62). Briefly, bacteria were harvested, washed twice with PBS, and diluted to approximately 10^8 CFU/ml. The suspensions were applied to empty wells of Lab-Tek II chamber slides (Nalge Nunc International, Naperville, Ill.), and unbound bacteria were removed by washing with PBS. The slides were blocked with a mixture of 1% gelatin and 10% normal donkey serum diluted in PBS. To detect Fba, the slides were successively incubated with Fba antiserum and donkey anti-rabbit IgG labeled with FITC. Neither strain 90-226 nor DC283 reacted with preimmune serum.

RESULTS

Growth phase effects on FH binding by GAS. We demonstrated previously that stationary-phase cultures of GAS strain 90-226 exhibit poor binding of FH (47). To determine if growth phase affects FH binding by other streptococcal isolates, we measured binding by exponential and stationary-phase cultures of GAS strains 80-003, 87-263, AP1, CS101, and MGAS5005 (Fig. 1). PCR analyses indicated that all of these GAS isolates carry *fba* (data not shown). With the exception of the negative control strain, DC283, exponential-phase GAS bound FH. Binding was reduced by an order of magnitude for stationary-phase cultures of strains 90-226 and 87-263 and to a lesser

FIG. 2. Western analysis of Fba expression. Cell surface proteins were extracted from exponential-phase cultures of the indicated bacterial strains. Fba protein was detected using Fba antiserum. Arrows indicate the migration positions of 97- and 45-kDa protein standards. The leftmost lane contains recombinant Fba protein (rFba). The calculated sizes of the mature Fba proteins, derived from the DNA sequences, are as follows: 30.2 kDa for 90-226 and MGAS5005; 32.7 kDa for 80-003 and 87-263; 33.8 kDa for AP1; and 33.5 kDa for CS101. Note that Fba proteins, whether extracted from streptococci or expressed as recombinant proteins, migrate anomalously on SDS-PAGE gels (62, 78). None of the protein bands evident in the figure reacted with preimmune serum.

extent for MGAS5005 and AP1. Growth phase did not affect FH binding by strain 80-003. As was previously noted for strain 90-226, all GAS isolates grown to stationary phase in the presence of the protease inhibitor E64 bound FH (61). Assay results for CS101 differed considerably between experiments. As a result, we were unable to make a determination regarding the effects of culture conditions on FH binding by CS101.

GAS binding of FH and FHL-1 is mediated by Fba protein. To determine if Fba contributes to FH and FHL-1 binding by the GAS isolates, *fba* was insertionally inactivated in each strain by transformation with pFW5Δ*fba*. Western analyses of cell surface proteins extracted from the *fba* mutants confirmed the absence of cell wall-anchored Fba (Fig. 2). Binding levels of FH and FHL-1 by the resulting mutants were then compared to those of the parental strains (Fig. 3). With the exception of CS101, disruption of *fba* inhibited binding of both ligands. In order to verify that the loss of FH and FHL-1 binding was due to inactivation of *fba* and not to an effect of the insertion on other genes, pYT1143 was introduced into each of the *fba* mutants. This plasmid carries the intact *fba* gene under control of the GAS *recA* promoter (78). The plasmid restored the capacities of the *fba* mutants to bind both ligands (Table 3), indicating that Fba is a major FH and FHL-1 binding protein of GAS strains 90-226, 80-003, 87-263, AP1, and MGAS5005. The *fba* derivative of CS101 did exhibit reduced binding of FHL-1 but did not significantly differ from the parent strain with regard to FH binding.

Strain 90-226 selectively binds FHL-1, rather than FH, when incubated with human plasma or serum, a property dependent on the expression of Fba protein (61). To determine if other Fba⁺ GAS exhibit similar properties, we performed plasma adsorption experiments with the GAS isolates and their isogenic *fba* derivatives (Fig. 3C). All six wild-type isolates bound more FHL-1 than FH, whereas none of the *fba* mutants bound detectable amounts of FHL-1. The low levels of FH bound by the wild-type strains were eliminated or reduced for the *fba* mutants.

SpeB is responsible for the loss of FH binding by stationary-phase GAS. The data presented in Fig. 3 demonstrate that Fba is a major FH and FHL-1 binding factor for serotype M1 GAS. This finding, in conjunction with the data in Fig. 1, suggested that Fba is subject to proteolytic degradation in some strains (e.g., 90-226 and 87-263) but not in others (e.g., 80-003). To determine if there was a correlation between the production of extracellular proteinase(s) and the loss of FH binding activity,

we measured proteinase activity in culture supernatants from stationary-phase cultures. We found that strains 90-226 and 87-263, strains that exhibited the lowest levels of FH binding in stationary phase, had the highest levels of proteinase activity (Fig. 4A). Strain 80-003 had the lowest level of proteinase activity, while strains AP1, CS101, and MGAS5005 produced low to intermediate levels of proteinases. The addition of 25 μM E64 to the assay mixtures inhibited the hydrolysis of azocasein by 85 to 96%, suggesting that most of the proteinase activity was due to SpeB. To compare amounts of SpeB produced by the different isolates, immunoblot analyses were performed with the same supernatants used to measure total protease activity (Fig. 4B). We found a good correlation between the amounts of the 28-kDa (proteolytically active) form of SpeB and proteinase levels in culture supernatants. Control experiments, in which blots were incubated with preimmune antiserum, confirmed that most of the bands present in the SpeB blots were not due to nonspecific binding of rabbit antibodies (data not shown). The exceptions were SpeB preparations from strain CS101, where bands ranging in size from 30 to 40 kDa reacted with both immune and normal rabbit sera. These bands probably represent immunoglobulin binding proteins known to be expressed by CS101 (41, 57). Collectively, these results suggested that SpeB accounted for the loss of Fba function in stationary-phase cultures.

To confirm and extend these findings, we constructed a *speB* mutant of strain 90-226. Culture supernatants of the mutant strain (DC349) had very low levels of protease activity (Fig. 4A) and no detectable SpeB protein (data not shown). In contrast to the results with the wild-type strain, neither growth phase nor the incorporation of E64 into culture media had an effect on FH or FHL-1 binding by DC349 (Fig. 5). To verify that SpeB affects cell surface expression of Fba, we extracted cell surface proteins from stationary-phase cultures of 90-226 and DC349, each grown in either the presence or the absence of E64. Immunoblot analyses were then performed for detection of Fba protein (Fig. 5C). As anticipated, we did not detect Fba in 90-226 extracts unless E64 had been added to the culture medium. In contrast, E64 had no effect on the amount of Fba extracted from strain DC349. Moreover, immunofluorescence microscopy confirmed that Fba was present on stationary-phase cells of DC349 but not on 90-226 or DC283 (Fig. 5D).

FH binding by recombinant Fba protein from GAS strain CS101. The data presented in Fig. 3B and C support the idea

FIG. 3. Inactivation of *fba* inhibits binding of FH and FHL-1. Binding of FH (A) and FHL-1 (B) was determined for each wild-type (Fba⁺) strain (hatched bars) or isogenic Fba⁻ derivative of each strain (gray bars). Bacterial cells were harvested from exponential-phase cultures and adsorbed to wells of microtiter plates. Binding assays were performed, and the results are presented as described in the legend to Fig. 1. (C) GAS binding of FH and FHL-1 from human plasma. GAS were incubated with human plasma, harvested by centrifugation, and washed with PBST. Bound plasma proteins were eluted and subjected to Western analysis using FH antiserum. The strain phenotypes are listed above the blots (+, wild type; —, mutant). The topmost arrow to the right of the blot indicates the 150-kDa FH band. The lower arrow indicates the 42-kDa band representing FHL-1. Numbers to the left of the blot indicate sizes of molecular mass markers (in kilodaltons).

that Fba mediates binding of FHL-1 by GAS strain CS101. We could not determine, however, whether Fba significantly contributes to FH binding by the same strain. This was due largely to the fact that the assay results for FH binding by CS101 were more variable than those for the other strains. It is important

to note that the size and amino acid sequence of the CS101 Fba protein (Fba_{CS101}) differs from those of the strain 90-226 protein (61, 70, 78). Therefore it seemed possible that Fba_{CS101} may bind to FHL-1 but not to FH. In order to test this possibility, a recombinant form of the CS101 Fba protein was purified and tested for binding activity. The results demonstrated that Fba_{CS101} binds both FH and FHL-1 (Fig. 6).

Sequence analyses of the Fba proteins from CS101 and 90-226 using the COILS program (version 2.2; see <http://www.ch.embnet.org/software/coils>) (52) indicated that both proteins have a putative, coiled-coil-forming region (Fig. 7). We previously reported that deletion of this region from the 90-226 protein resulted in a loss of FH and FHL-1 binding (62). To determine if the analogous region of Fba_{CS101} is important for ligand binding, we tested a recombinant protein lacking this region (Fba_{CS101}Δ71-125) for binding of FH and FHL-1 (Fig. 6). The deletion significantly reduced binding of both ligands. To verify that the loss of binding was not simply due to an effect of the deletion on the structure of the protein, we expressed amino acid residues 71 to 125 of Fba_{CS101} as a GST fusion protein (Fba_{CS101}71-125). As a control, a similar construct, Fba₉₀₋₂₂₆69-112, was made for expression of the FH and FHL-1 binding region of the strain 90-226 protein. Both proteins were capable of binding FH and FHL-1, thus confirming that the region of Fba_{CS101} encompassing amino acid residues 69 to 125 contains the major FH and FHL-1 binding site (Fig. 8).

DISCUSSION

In earlier studies, we found that Fba is important for recruitment of FHL-1 and FH by the serotype M1 isolate 90-226. In the present study, we examined the role of Fba in acquisition of FH and FHL-1 by four additional M1 isolates. These organisms were isolated from patients with uncomplicated pharyngitis (strains 80-003 and 87-263) and invasive disease (strains 90-226 and MGAS5005) and were originally isolated in Europe and North America between 1980 and the late 1990s. Thus,

TABLE 3. Genetic complementation of *fba* mutations by pYT1143

Parental strain	Fba ^a	pYT1143 ^a	Ligand binding ^b	
			FH	FHL-1
90-226	+	—	1.043	0.897
80-003	+	—	0.939	0.538
	—	—	0.083	0.133
	—	+	1.147	0.816
87-263	+	—	0.876	0.834
	—	—	0.052	0.081
	—	+	0.991	0.996
AP1	+	—	1.137	1.086
	—	—	0.125	0.117
	—	+	1.262	0.727
MGAS5005	+	—	0.855	1.070
	—	—	0.073	0.143
	—	+	0.845	0.806

^a +, present; —, absent.

^b Values are average absorbancies at 405 nm from results for a single experiment in which each strain was assayed four times.

FIG. 4. SpeB expression by GAS isolates. GAS were cultured for 16 h in THY medium or THY medium containing E64 (90-226 + E64). The OD₆₀₀ (Abs₆₀₀) of each culture was determined, and the culture supernatants were filter sterilized. (A) Proteolytic activity in culture supernatants. Assays were performed as previously described using azocasein as the substrate (2, 11). Data are from a representative experiment in which each assay was performed four times. Experiments were performed three times. (B) SpeB in culture supernatants. Proteins in culture supernatants were separated by SDS-PAGE and blotted onto polyvinylidene difluoride membranes. SpeB was detected with SpeB antiserum and a labeled secondary antibody. Migration of 28.9- and 52.1-kDa protein standards are indicated on the right. SpeB, purified SpeB protein.

they represent a diverse set of isolates (10–12, 35, 44). GAS strain CS101 is a well-characterized M49 isolate (41, 70). Genetic inactivation of *fba* greatly inhibited binding of purified FH and FHL-1 by each serotype M1 strain, indicating that Fba is a major FH and FHL-1 binding factor of this GAS serotype. Similarly, the *fba* mutants failed to bind appreciable amounts of FH or FHL-1 from human plasma.

We attempted to determine, by use of genetic methods, whether Fba is a major FH and/or FHL-1 protein of GAS strain CS101. This was of particular interest because whereas the FH and FHL-1 binding site of Fba₉₀₋₂₂₆ is represented by the sequence YKQIKTAPDKDKLLF, the corresponding sequence in Fba_{CS101} is YKQIDAETDKDKLLL (62, 70). We found that the *fba* derivative of CS101 bound FHL-1 less well than the parental strain, but our results with regard to the role of Fba_{CS101} in FH binding were inconclusive. The inconclusiveness was due, in part, to the fact that binding results were more variable for CS101 than for the other GAS strains, thus making it difficult to obtain statistically significant data. To establish whether Fba_{CS101} can bind FH, we tested a recombinant form of the protein for binding of FH (and FHL-1).

Fba_{CS101} was found to bind both ligands but with reduced affinities relative to the binding of the ligands by Fba₉₀₋₂₂₆. We conclude that Fba is a major FHL-1 binding factor of CS101 and that Fba contributes to FH binding.

Deletion analysis of Fba₉₀₋₂₂₆ localized the binding site for FHL-1 and FH to a 36-amino-acid, putative coiled-coil region of the protein. PepSpot analyses confirmed that a linear domain that overlaps the coiled-coil region mediates binding to FHL-1 and to FH (62). Although there are differences in the *fba* genes among the isolates, the ligand binding site identified in the strain 90-226 protein is conserved in the serotype M1 isolates used in this study. This region is not strictly conserved in Fba_{CS101}, however (70, 78). Our initial analysis of the CS101 Fba protein revealed the presence of a 54-amino-acid, putative coiled-coil encompassing residues 72 to 125. A recombinant protein with this region deleted failed to bind either FH or FHL-1, suggesting that the ligand binding site is within this portion of the protein. This possibility was confirmed by our finding that recombinant proteins representing the putative coiled-coil regions of Fba₉₀₋₂₂₆ and Fba_{CS101} both bound to FH and FHL-1. It is important to note that while Fba is predicted to possess a coiled-coil region, this region of the protein is enriched in charged amino acids which can produce false-positive probabilities for formation of coiled-coils (51). While additional studies are required to precisely define the nature of the ligand binding site, the present studies demonstrate that FHL-1 and FH binding is a conserved function of the putative coiled-coil region of Fba.

We previously reported that the capacity of strain 90-226 to bind FH was largely abolished in stationary-phase cultures. Moreover, the loss of FH binding correlated with the absence of Fba on the cell surface. Because streptococci grown in the presence of E64 retained FH binding capacity in stationary phase, we speculated that SpeB was likely involved in degradation or cleavage of Fba. SpeB is known to affect the cell surface expression of several GAS proteins, including M protein and C5a peptidase (4, 7, 72). It has been proposed that proteolysis of GAS cell surface proteins may facilitate bacterial detachment from tissues and subsequent dissemination to uninfected sites once nutrients become limiting at the primary site of infection (74). Although we suspected that SpeB was responsible for the release of Fba from GAS, it has been shown that many GAS isolates do not produce appreciable amounts of SpeB when cultured in THY medium, the medium used in our previous and present studies (8, 12). Therefore, it was possible that an unknown protease was involved in the release of Fba from the cell surface. In the present study, we observed a wide variation in the capacities of stationary-phase GAS to bind FH. For most isolates, the capacity of stationary-phase cells to bind FH correlated with low levels of proteinase activity and SpeB protein in culture supernatants. The role of SpeB in degradation of Fba was confirmed by inactivation of *speB* in strain 90-226. In contrast to the wild-type parental strain, stationary-phase cultures of the *speB* mutant had Fba on the cell surface and bound FH as well as exponential-phase streptococci did. Our results demonstrate that cell surface expression of Fba varies considerably between isolates of the same serotype and that the variability is due to variations in the level of SpeB production. Thus, strain 90-226 is neither a unique nor a

FIG. 5. Inactivation of *speB* affects binding of FH and FHL-1 and cell surface expression of Fba protein. FH (A) and FHL-1 (B) binding by *SpeB*⁺ and *SpeB*⁻ GAS. Ligand binding was measured for GAS grown to mid-exponential phase (hatched bars), stationary phase (gray bars), or stationary phase in the presence of E64 (open bars). Assays were performed and results are presented as described in the legend to Fig. 1. (C) Western analysis of Fba expression. Cell surface proteins were extracted from stationary-phase cultures of 90-226 (*Fba*⁺ *SpeB*⁺), DC349 (*Fba*⁺ *SpeB*⁻), and DC283 (*Fba*⁻ *SpeB*⁺). Streptococci used in the experiment were grown in either THY medium (lanes labeled -) or THY medium containing E64 (lanes labeled +). Blots were probed with Fba antiserum. rFba, purified recombinant Fba protein. (D) Immunofluorescence microscopy for detection of Fba protein. GAS strains 90-226 (i and ii), DC349 (iii), and DC283 (iv) were harvested from exponential-phase (i) or stationary-phase (ii to iv) cultures and applied to chamber slides. Slides were incubated with Fba antiserum and an FITC-labeled secondary antibody.

representative serotype M1 strain with regard to stationary-phase expression of Fba.

Nearly intact molecules of M1 protein and protein H, two highly expressed cell surface proteins, have been detected in culture supernatants of the AP1 strain of GAS (4, 33). Because the soluble forms of these proteins seem capable of contributing to immune evasion, we were intrigued with the possibility that Fba might accumulate in culture supernatants. We did not detect Fba in culture supernatants of any of the strains examined in this study, which suggests that any Fba released from the cell surface is subject to degradation. Moreover, *SpeB* was found to completely degrade purified Fba protein (data not shown). Obviously, our *in vitro* experiments do not directly reflect *in vivo* conditions, where streptococci are likely to be coated with host proteins that could protect bacterial proteins from *SpeB*, but they do suggest that significant amounts of Fba are unlikely to be released from GAS during infection.

It is not clear what factors account for high expression levels of *SpeB* by some isolates, e.g., 90-226, and low expression levels by other isolates (e.g., AP1). The fact that production of *SpeB* can be induced by growing the AP1 strain in a specialized medium suggests that *SpeB* may be subject to different regulatory mechanisms in different strains. Expression of *fba* is controlled by the positive transcriptional regulator Mga (70, 78). Mga-regulated genes, which include *emm*, *sic*, and *scpA*, are transcribed in the exponential phase of growth (56, 57). *SpeB*, on the other hand, is produced in early stationary phase (8, 9, 69). The regulation of *SpeB* production and activity is surprisingly complex. Expression of *speB*, as well as numerous other virulence factors, requires the transcriptional activator Rgg/RpoB (9, 53, 54). The *speB* gene is also subject to negative regulation by CsrRS/CovRS, a two-component system that affects transcription of 15% of all GAS genes (23, 29, 30, 31, 66). Other factors known to influence *speB* expression are oligo-

FIG. 6. Binding of FH and FHL-1 by recombinant Fba proteins. (A) FH binding by wild-type and mutant Fba proteins. Recombinant proteins representing the wild-type proteins from GAS strains CS101 (Fba_{CS101} 38-348) and 90-226 (Fba₉₀₋₂₂₆ 37-324) and derivatives were adsorbed to microtiter plates and incubated with various concentrations of FH. Binding was detected with FH antiserum. (B) FHL-1 binding by the same recombinant proteins described for panel A. Each panel shows the results of a representative experiment in which each assay was performed three times. Bars represent ranges. Abs₄₀₅, absorbance at 405 nm.

peptide and dipeptide permeases (67, 66); *pelA* or *sagA*, which are involved in the production of streptolysin S (5, 50); *luxA*, a gene required for production of autoinducer II (55); and Nra and its homologues (58). Secretion and processing of SpeB de-

pend on expression of trigger factor (peptidyl-prolyl *cis-trans*-isomerase) (53, 54) and cell surface expression of M protein (12). In addition, transcription of *speB* is upregulated by phagocytosis of GAS by polymorphonuclear leukocytes (79).

Despite a wealth of information on the regulation of SpeB, the signals governing expression during infection and the role of SpeB in GAS pathogenesis are not fully understood. The impact of cell surface proteolysis on pathogenesis is also unclear. Aziz et al. (2) found, by using an *in vivo* tissue chamber model of infection, that SpeB expression can result in the degradation of several immune evasion proteins, including the cell surface M1 protein. Moreover, these authors reported that downregulation of SpeB is apparently important to maintain infection, at least under conditions where GAS dissemination within a host is blocked. Rasmussen and Björck (74) have proposed a two-phase model of the possible role of cell surface proteolysis in GAS pathogenesis. The first phase, corresponding roughly to logarithmic growth, is associated with expression of cell surface proteins, such as M, C5a peptidase, and Fba,

FIG. 7. Predicted coiled-coil regions in Fba proteins from strains 90-226 and CS101. Probabilities of coiled-coil formation for Fba₉₀₋₂₂₆ (A) and Fba_{CS101} (B) were determined with the COILS program (version 2.2; see www.ch.embnet.org/software/coils) (52) using window sizes of 14, 21, and 28 amino acid residues, as indicated, with weighting in favor of hydrophobic residues at the *a* and *d* positions of heptad repeats.

FIG. 8. Localization of the ligand binding site in Fba_{CS101}. Binding of FH (open symbols) and FHL-1 (filled symbols) by Fba_{CS101} 71-125 (triangles), Fba₉₀₋₂₂₆ 69-112 (circles), and GST (squares). Results of a representative experiment are shown.

that downregulate complement deposition and with a low level of cell surface proteolysis. SpeB is expressed in the second phase of infection, when the population of GAS at infected sites is high and nutrients are less available. Proteolytic activity in the second phase is proposed to degrade proteins involved in the formation of bacterial aggregates, freeing GAS to disseminate to uninfected sites. The M1 and Fba proteins both contribute to GAS resistance to phagocytosis and invasion of host cells (14, 62, 78). The fact that both proteins are subject to SpeB cleavage indicates that cell surface proteolysis has the potential to have a significant impact on GAS-host interactions during infection.

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