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The Signal Recognition Particle Pathway Is Required for Virulence in *Streptococcus pyogenes*▽

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The signal recognition particle (SRP) pathway is a universally conserved pathway for targeting polypeptides for secretion via the cotranslational pathway. In particular, the SRP pathway is thought to be the main mechanism for targeting polypeptides in gram-positive bacteria, including a number of important human pathogens. Though widely considered to be an essential cellular component, recent advances have indicated this pathway may be dispensable in gram-positive bacteria of the genus *Streptococcus* under in vitro conditions. However, its importance for the pathogenesis of streptococcal disease is unknown. In this study, we investigated the importance of the SRP pathway for virulence factor secretion in the human pathogen *Streptococcus pyogenes*. While the SRP pathway was not found to be essential for viability in vitro, SRP mutants demonstrated a medium-specific growth defect that could be rescued by the addition of glucose. We also observed that a distinct subset of virulence factors were dependent upon the SRP pathway for secretion, whereas others were completely independent of this pathway. Significantly, deletion of the SRP pathway resulted in mutants that were highly attenuated in both a zebrafish model of necrotic myositis and a murine subcutaneous ulcer model, highlighting the importance of this pathway in vivo. These studies emphasize the importance of the SRP pathway for the in vivo survival and pathogenesis of *S. pyogenes*.

The important human pathogen *Streptococcus pyogenes* is able to establish infection and cause disease in a wide variety of tissues in the host, including the pharynx (e.g., "strep throat") and the epidermis (e.g., impetigo and erysipelas), as well as deeper tissues like the dermis, fascia, and muscle (e.g., cellulitis, necrotizing fasciitis, and myositis). Crucial to the establishment of infection is the production of a multitude of virulence factors that are secreted across the single cellular membrane of this gram-positive pathogen (14). Understanding how these various factors are trafficked to their appropriate destinations, including the streptococcal cell membrane, its cell surface, the extracellular spaces, and host cells, is important for elucidating the role of protein secretion in streptococcal virulence. An unexplored question is how intracellular routing of a nascent polypeptide contributes to its trafficking fate.

Protein secretion by gram-positive bacteria has recently been revealed to be a complex, organized process with specific domains dedicated to protein secretion and folding (11, 42, 43). However, the pathways used to route presecretory proteins to the sites of translocation across the membrane are not well understood. As gram-positive bacteria lack a discernible homolog of SecB, a chaperone involved in the posttranslational targeting pathway, most polypeptides are presumably targeted cotranslationally via the signal recognition particle (SRP) pathway (45). This is supported by the fact that most gram-positive signal sequences tend to be longer and more hydrophobic than their gram-negative counterparts, which are features thought to be important for efficient routing of the presecretory protein to the SRP pathway (12). Many studies of model bacterial species, including *Escherichia coli* and *Bacillus subtilis*, have highlighted the essential nature of the SRP pathway for bacterial viability (25, 41). However, more recent studies of bacteria of the genus *Streptococcus* have indicated that SRP essentiality may not be a universal characteristic of all bacteria (23).

The SRP itself is a ribonucleotide-protein complex composed of a protein, the fifty-four homolog (Ffh), and an RNA known as the small cytoplasmic RNA (scRNA) (45). The SRP binds to signal sequences as they exit the ribosome and targets them to the bacterial membrane by binding the membrane-associated receptor FtsY, which then releases the signal peptide to the Sec translocon (30). Recent cryoelectron micrographs revealed the details behind this process whereby the interaction between FtsY with the ribosome and Ffh results in a conformational change in the ribosome to promote binding to the translocation machinery (21). Crucial for proper function of SRP are the GTPase activities of both Ffh and FtsY, which are shared in a common catalytic chamber of this heterodimeric complex (16). The SRP receptor interacts directly with the SecYEG translocon, highlighting the close association between these two protein complexes (3).

Interestingly, deletion of the genes encoding Ffh, FtsY, and scRNA singly or in combination in *Streptococcus mutans* is not a lethal event for growth on complex media (23). Under nonstress conditions, the SRP$^-$ mutants have growth yields similar to that of the wild type, although with somewhat longer doubling times during logarithmic growth (23). The SRP mutants

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do have a reduced capacity to resist certain stresses, most prominently osmotic and acid shock (20), and fail to form biofilm (24). However, a decreased resistance to stress is not a global phenotype, as the mutants do retain an ability to adapt to acidic environments under conditions that more gradually expose them to decreased pH (13). Also, their growth patterns are not altered in response to many other stresses, including high and low temperatures (23). The importance of the SRP for the pathogenesis of *S. mutans* disease remains to be determined.

Studies of *E. coli* have suggested that the SRP pathway is the major pathway for targeting secretion of integral membrane proteins, which typically lack cleavable signal sequences (44). Similarly, analysis of membranes of *S. mutans* SRP^- mutants demonstrated that approximately 17 proteins either were absent or were present at significantly reduced levels (24). The latter class included the β-subunit of the F_1F_0 ATPase, which is known to be a SRP substrate in other bacterial species (47). Since this proton pump plays a central role in the ability to adapt to acidic conditions (5), this defect may explain the enhanced sensitivity of these mutants to acid stress. Other proteins with altered membrane abundance included several glycolytic enzymes and LuxS, which is responsible for synthesis of an autoinducer known to be important for formation of streptococcal biofilm (46, 48). However, most of these proteins lack clearly identifiable signal sequences, so the global importance of the SRP for the trafficking of polypeptides with both cleavable and noncleavable signal sequences in the streptococci is unknown.

The objectives of the present study were to examine whether the nonessentiality of the SRP can be generalized to other pathogenic streptococcal species, and if so, what role the SRP pathway may play in the secretion of virulence factors and whether the SRP is required for virulence. Our analysis of an Ffh-deficient mutant of *S. pyogenes* revealed that a distinct subset of virulence factors with cleavable signal sequences was dependent upon the SRP pathway for secretion. Furthermore, SRP^- mutants were highly attenuated in animal models of necrotic myositis and subcutaneous infection. These data provide insight into the conservation of the SRP pathway function in the streptococci and suggest that, while it is not essential for growth, it is essential for virulence.

MATERIALS AND METHODS

Strains, media, and culture conditions. Strains used included *E. coli* DH5α and *S. pyogenes* HSC5 (22). Unless otherwise indicated, routine culture of *S. pyogenes* was done at 37°C and employed Todd-Hewitt medium (BBL) supplemented with 0.2% yeast extract (Difco). For certain experiments, *S. pyogenes* was cultured in C medium under conditions previously described (32). Culture of *E. coli* utilized Luria-Bertani (LB) broth at 37°C with shaking. When required, antibiotics were used at the following concentrations: erythromycin at 750 μg/ml for *E. coli* and 1 μg/ml for *S. pyogenes*.

DNA and computational techniques. Plasmid DNA was isolated via standard techniques and used to transform *S. pyogenes* as described previously (10). Restriction endonucleases, ligases, and polymerases were used according to the manufacturer's recommendations. The fidelity of all constructs derived by PCR was confirmed by DNA sequencing analyses. All references to genomic loci are based on the genome of *S. pyogenes* strain SF370 (15). Gene assignments were based on the information available in the Kyoto Encyclopedia of Genes and Genomes (www.genome.jp) and were supported by subsequent interrogation of the SF370 genome using BLAST (2) and query sequences derived from *Bacillus subtilis* gene products with experimentally confirmed activities, as noted in the text.

Construction of mutants. An in-frame deletion was constructed by an inverse-PCR technique (31) to delete an internal fragment encompassing 500 bp of the central portion of *ffh* (SPy_1200). The next downstream open reading frame (SPy_1198) is transcribed from the strand opposite *ffh*, suggesting that deletion mutations in *ffh* will not be polar. Allelic replacement proceeded as described previously (31), with confirmation of mutant genome structure by PCR using primers of the appropriate sequences. Primers for the deletion included Ffh deletion 1 (AAC CTA GGG GGC CTA TGG AAG ACC TCT TGA AAA TGA TTC CAG G) and Ffh deletion 2 (AAC CTA GGC AAA CCT TTT CTG ACA ATA TCG ACT GC). The resulting fragment of *ffh* has a deletion from amino acids spanning positions 180 to 340. The method for allelic replacement involves the construction of a merodiploid strain that contains both mutant and wild-type alleles in tandem on the chromosome that resolves to contain either the mutant or wild-type alleles (9, 31). Thus, to control for any unanticipated secondary mutations that may have arisen during the production of the mutant, all comparisons were made between a mutant and a matched wild-type sibling that arose from the same merodiploid.

Cellular fractionation and preparation of membranes. Protoplasts were prepared as previously described (40) and then lysed by multiple freeze-thaw cycles (−80°C/37°C) followed by agitation with glass beads (106 μm; Sigma) using a reciprocating shaking device (FastPrep; Qbiogene) at a speed setting of 4.5 for 45 s, repeated four to five times. Membranes were collected by centrifugation (120,000 × *g*, 4°C, 60 min) and resuspended in distilled water. Sodium dodecyl sulfate sample buffer was added to the membranes, followed by a 10-min boil. Membrane fractions were then separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), transferred to an Immobilon-PSQ membrane, and stained with Coomassie brilliant blue R. Protein identification was performed by N-terminal sequencing of Coomassie-stained bands (Midwest Analytical, St. Louis, MO).

Analysis and identification of proteins. Cell wall, membrane, and cytosolic fractions were prepared from cultures grown in Todd-Hewitt (THY) broth as previously described (43). Cell-free supernatants were subjected to trichloroacetic acid (TCA) precipitation for protein concentration. Cell wall fractions from protoplast preparations were utilized for M protein analysis, while culture supernatants were utilized for analysis of SpeB, streptolysin O (SLO), and *S. pyogenes* NAD glycohydrolase (SPN). Other fractions were consistently negative for the presence of these proteins. Protein samples were separated by SDS-PAGE and transferred to polyvinylidene difluoride membranes. Blocking and antibody binding were done in phosphate-buffered saline–Tween 20 with 5% nonfat dry milk. Antisera against SLO and SpeB have been described previously (31, 35). An antipeptide antiserum that recognizes multiple serotypes of M protein was generated as described previously (17). An antiserum that reacts specifically against SPN was generated using purified SPN (C. Smith, M. Caparon, and S. Hultgren, unpublished). Analyses of culture supernatant fluids for SpeB proteolytic activity and SLO hemolytic activity were conducted as described previously (32, 35). Relative protein concentrations were quantitated from digitized images of Western blots by using ImageJ software (http://rsb.info.nih.gov/ij/). Data are expressed as the ratio of mutant versus wild type and were derived from a minimum of three independent experiments.

Analysis of transcription. RNA from various streptococcal strains was isolated and analyzed as described previously (7, 29). Briefly, strains grown overnight in THY medium were diluted 1:100 in fresh THY medium, followed by growth at 37°C to mid-logarithmic phase (optical density at 600 nm [OD_{600}] of 0.500). RNA was then isolated by using glass beads (Lysing Matrix A; Qbiogene) and a high-speed reciprocating shaking device (FP-120; Savant Instruments). RNA was further purified (RNeasy mini kit; Qiagen) and contaminating DNA was removed by DNase treatments according to the manufacturer's instructions (RNase-free DNase set [Qiagen] and DNase I, amplification grade [Invitrogen]). The A_{260}/A_{280} ratio was used to determine the RNA concentration and purity. For cDNA synthesis, 5 μg total RNA was treated with 200 U Superscript II reverse transcriptase (Invitrogen) using 250 ng of oligonucleotide random primers (Invitrogen). Real-time reverse transcription-PCR (RT-PCR) was performed with an iCycler thermocycler (Bio-Rad) using iQ Sybr green supermix (Bio-Rad) and methods and primers described previously (29). Transcript abundance was normalized to that of *recA* as previously described (29), and data presented represent the means and standard deviations derived from at least three independent experiments that were performed on different days, with each individual sample analyzed in triplicate.

Zebrafish infections. Bacterial strains were back diluted 1:100 from overnight culture and grown in THY broth to an OD_{600} of 0.30. Bacteria were then subjected to brief sonication to disrupt bacterial chains and used for intramuscular injection of zebrafish, as described previously (39). Experimental groups consisted of 10 zebrafish, each of which was injected with 10^5 CFU in a volume

of 10 μl or, in the case of mock infection, with an equivalent volume of sterile medium alone. Survival was monitored for 5 days, and any differences in survival between groups infected with wild-type or mutant strains were tested for significance as described previously (7). Data presented are pooled from three independent experiments that were conducted on different days.

Mouse subcutaneous infections. Bacterial strains were grown and prepared as outlined above and then used to infect mice subcutaneously as previously described (8). Experimental groups consisted of five mice, each of which received a subcutaneous injection of 10^8 CFU in a volume of 100 μl into the right flank. Lesion development was monitored over a period of 96 h and recorded as described elsewhere (8). Differences between groups in lesion area and in number of mice developing an ulcer were tested for significance by the Mann-Whitney U test and the chi-square test with Yates' correction, respectively (8). For all test statistics, the null hypothesis was rejected when the P value was <0.05. Data presented are pooled from two independent experiments that were conducted on different days.

RESULTS

Ffh is not essential in *Streptococcus pyogenes*. Disruption of the SRP pathway leads to a variety of phenotypes in the various bacterial species examined. In *E. coli* and *B. subtilis*, deletion of Ffh is lethal (41). Furthermore, conditional depletion of Ffh and FtsY in *B. subtilis* results in a marked impairment of protein secretion (25), although the effect on the secretome may be more modest when the bacteria are cultured in rich media (49). In *Streptococcus mutans*, disruption of Ffh was first revealed in a screen for acid-sensitive mutants (19). It was subsequently shown that all components of the SRP pathway could be disrupted extensively in *S. mutans* with few phenotypic consequences apart from sensitivity to certain stresses (13). Interestingly, disruption of both the SRP and one of its two homologs of YidC (YidC2) resulted in *S. mutans* cells which were severely impaired in growth (23). We sought to ascertain whether SRP was also dispensable for the growth of *S. pyogenes* and to determine the effect of SRP disruption on the secretion of several important virulence factors. The SRP includes three critical components, a protein (Ffh), an RNA (scRNA), and a membrane receptor (FtsY) (45), and it has been shown that mutation of the genes encoding any of the components results in the same stress-sensitive phenotype in *S. mutans* (23). For analysis of *S. pyogenes*, we constructed an in-frame deletion in the allele of the gene encoding Ffh that removed 500 bp from the central portion of the Ffh open reading frame, which includes the region encoding conserved residues necessary for GTPase activity that are crucial for function (16). The resulting mutation in the allele [*ffh*(Δ180–340)] was successfully used to replace the wild-type gene in *S. pyogenes* HSC5 (Fig. 1A), and the resulting mutants grew normally and produced colonies on THY agar plates that were indistinguishable from wild-type colonies. Thus, similarly to results for *S. mutans*, the SRP pathway is dispensable for viability of *S. pyogenes*.

Medium-specific growth defect. The method for allelic replacement generates a tandem duplication of wild-type and mutant alleles that resolves by homologous recombination to produce strains carrying either the wild-type or the mutant allele (9). One Ffh$^-$ mutant (FFH1) was chosen for further analysis, and its ability to grow in liquid media under various nutrient conditions was compared with that of a matched wild-type revertant (WT_{rev}). When grown in standard THY broth, a complex medium rich in glucose (18), the mutant showed a modest defect in its growth rate but obtained a growth yield

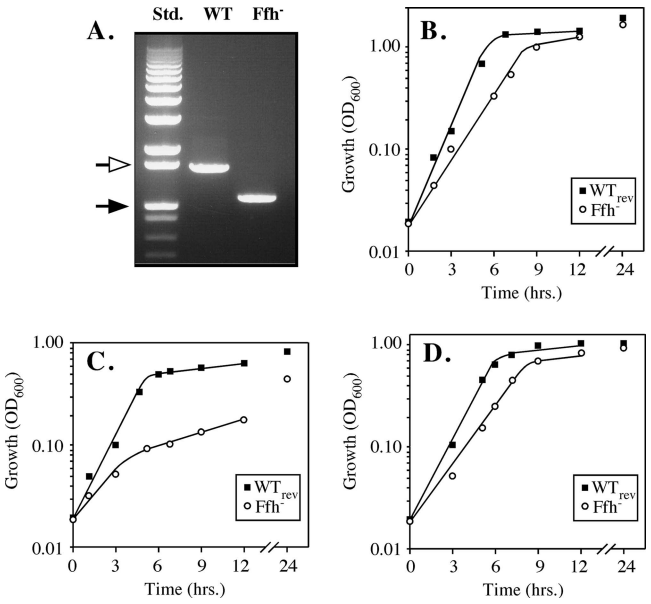

FIG. 1. Deletion of *ffh* results in specific nutritional requirements. Successful replacement of *ffh* (SPy_1200) in the wild-type strain with a deletion in the allele [*ffh*(Δ180–340)] produced Ffh$^-$ strain FFH1. (A) Analysis of PCR products generated using *ffh*-specific primers by agarose gel electrophoresis revealed bands of the expected sizes for the wild-type (WT, open arrow) and mutant (Ffh$^-$, filled arrow) strains as shown. Open and filled arrows at the left of the DNA size standards (Std.) (TrackIt DNA ladder; Invitrogen) indicate the 1,650- and 1,000-bp bands, respectively. The growth of the mutant (Ffh$^-$) in liquid media was compared to that of a matched wild-type revertant (WT_{rev}) in THY medium (B), C medium (C), and C medium supplemented with glucose (D). Data shown are from a single experiment representative of a minimum of three independent experiments conducted on different days.

equivalent to that of the wild type on the basis of culture densities (Fig. 1B). When grown in C medium, a more minimal medium rich in peptides but low in glucose, the Ffh$^-$ mutant presented a pronounced defect in both growth rate and yield, with cultures reaching a final density that was less that 50% of that obtained with a strain with the wild-type gene (Fig. 1C). However, this defect could be rescued by the addition of more glucose to C medium, which produced a growth profile that was indistinguishable from that of THY medium (Fig. 1, compare panels D and B). Addition of NaCl (up to 150 mM) did not restore growth; however, buffering the pH of the medium to between pH 6.0 and pH 6.5 had the same effect as the addition of glucose and restored growth of the mutant to levels identical to those achieved with THY medium (data not shown).

Stress response activated. Membrane protein profiles of the wild-type revertant and the Ffh$^-$ mutant were compared. Consistent with the relatively normal growth characteristics of the mutant in THY broth, these profiles were remarkably similar (Fig. 2). An exception was the presence of two bands that were consistently more intense in membranes of the mutant than in those of the wild-type revertant. Subjecting these bands to N-terminal sequencing revealed them to be DnaK and GroEL (Fig. 2). These proteins play prominent roles in several stress responses, particularly under conditions where misfolded pro-

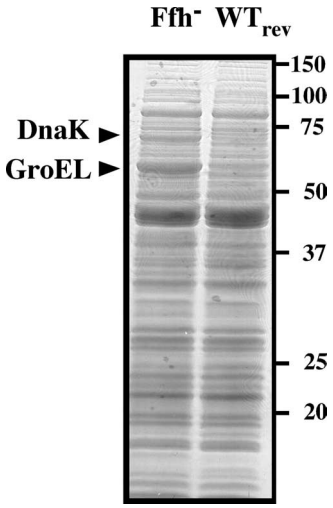

FIG. 2. The majority of membrane proteins are targeted correctly in the absence of the SRP. Membranes purified from a mutant (Ffh$^-$) and a matched wild-type revertant (WT_{rev}) were analyzed by SDS-PAGE and staining with Coomassie blue as shown. The identities of the bands labeled on the left of the figure were determined by N-terminal amino acid sequencing. The migration of several size standards (in kilodaltons) is indicated to the right of the figure.

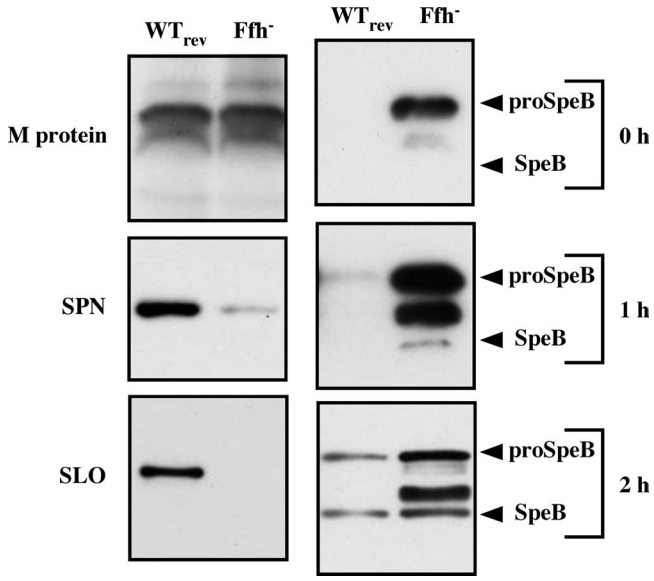

FIG. 3. The SRP is required for the secretion of a distinct subset of virulence factors. Analysis of the secretion of a selection of virulence factors that have both cleavable signal sequences and distinct trafficking fates during infection is shown. Included are the sortase pathway substrate M protein, the SpeB cysteine protease that is released into the extracellular compartment, SLO that is trafficked to the host cell membrane, and SPN that is translocated into the host cell cytosol. The mutant (Ffh$^-$) and a matched wild-type revertant (WT_{rev}) were compared by Western blot analyses using antisera specific for each indicated protein. Samples analyzed for M protein were cell walls from protoplast preparations, cell-free overnight culture supernatant fluids for SpeB, and cell-free overnight supernatant fluids that were concentrated by TCA precipitation for SLO and SPN. The migration of the zymogen (proSpeB) and mature (SpeB) forms of SpeB are indicated to the right of the SpeB panel. The times shown on the right of the figure indicate the onset of stationary phase (0 h) and subsequent time points.

teins accumulate (38, 49). This observation suggests that, despite near-normal growth characteristics in this medium, the mutant is experiencing stress or may be accumulating some misfolded proteins.

Selective defects in secretion of proteins with cleavable signal sequences. A prominent characteristic of *S. pyogenes* is its ability to secrete a large number of proteins during growth in culture and in tissues that have cleavable signal sequences. The fate of a subset of these proteins, including the M protein, is to become covalently associated with the cell wall (34). Others have well-characterized toxic activities and are known to have distinctly different trafficking fates when the bacterium is growing while attached to the surface of host cells. For example, many secreted proteins, such as the SpeB cysteine protease, are trafficked into the extracellular spaces. At least one, SPN (also known as Nga), is translocated across the host cell membrane into its cytosol by a process that involves a second secreted translocator protein, SLO, that is itself delivered to the host cell membrane (33, 35, 37). Since M protein, SpeB, SPN, and SLO have different trafficking fates, the consequence of the loss of Ffh on their secretion was assessed. Following secretion of the M protein via the Sec pathway, the signal sequence of the M protein is cleaved and subsequently processed at a site toward its carboxy-terminal end and covalently cross-linked to peptidoglycan by the enzyme sortase (4). Examination of cell walls revealed that the amount of M protein in the mutant was similar to or smaller than the amount of M protein in the wild-type parental strain (Fig. 3), indicating that Ffh is not essential for the secretion of M protein and its delivery to the sortase pathway. In contrast, the loss of Ffh had marked effects on the secretion of SPN and SLO. Analysis of culture supernatants revealed that SPN and SLO were undetectable or present at very low levels even after the proteins in these supernatants were concentrated by TCA precipitation (Fig. 3). Additionally, culture supernatants from Ffh$^-$ mutants lacked detectable SLO hemolytic activity and SPN activity (Table 1), confirming a defect for secretion of these proteins. The genes encoding SPN and SLO are cotranscribed from a common promoter (36), and the failure to observe the secreted forms of either protein was not due to a defect in transcription from this promoter, as analysis of the *slo* and *spn* transcripts by real-time RT-PCR at the time of maximal expression in logarithmically growing cultures indicated that these transcripts were as abundant in the mutant as in the wild-type revertant (Table 1). A similar analysis of the transcript levels of the gene encoding M protein at a time point corresponding to the period of maximal expression also showed no differences between the Ffh$^-$ mutant strain and the wild-type revertant (Table 1). No intracellular pool of SLO was detected when the mutant was subjected to lysis at this time point (data not shown), suggesting that the polypeptide was degraded rapidly in the absence of secretion, although translational arrest of the message could also account for this observation. Taken together, these data show that the SRP pathway does make a critical contribution to secretion of at least a subset of the exported virulence factors of *S. pyogenes* that possess cleavable signal sequences.

Medium-dependent effect on secretion of SpeB. The absence of Ffh had a different effect on secretion of SpeB, as it was

TABLE 1. Comparison of virulence factor expression levels between wild-type and Ffh$^-$ *S. pyogenes* strains

Virulence factor	Growth phase[a]	Gene transcript abundance (ratio)[b]	Protein expression (%)[c]	Activity (%)[d]
M protein	Late log	0.78 ± 0.20	101 ± 22	NT[e]
SpeB	Late log	97.3 ± 84	ND[f]	NT
	Stat (t = 0 h)	2.0 ± 0.4	1,000[g]	NT
	Stat (t = 1 h)	1.4 ± 0.8	1,464 ± 395	NT
	Stat (t = 2 h)	0.19 ± 0.1	928 ± 22	64 ± 3.4
SLO	Late log	1.02 ± 0.12	ND	ND
SPN	Late log	0.80 ± 0.24	11 ± 8.7	ND

[a] Growth in THY broth was monitored by OD_{600} for analysis at the stages noted, including the onset of stationary (Stat) phase (t = 0 h) and several subsequent time points.
[b] Relative transcript abundance of the genes encoding the listed virulence factors was determined by real-time RT-PCR and is expressed as the ratio of Ffh$^-$/WT_{rev}.
[c] Relative protein expression was determined by densitometric analyses of Western blots and is expressed as the percentage of Ffh$^-$ versus WT_{rev}.
[d] Activities tested were as follows: for SpeB, cysteine protease activity; for SLO, hemolytic activity; and for SPN, NAD glycohydrolase activity.
[e] NT, not tested.
[f] ND, not detected.
[g] The protein level in WT_{rev} was undetectable. A minimum estimate is presented.

observed that the Ffh$^-$ mutant secreted the SpeB polypeptide at levels higher than those observed for the wild-type revertant during growth in THY medium (Fig. 3). Much of this effect resulted from an alteration in the temporal pattern of *speB* transcription. In the wild type, *speB* is not transcribed during the log phase of growth, and transcription is initiated at high levels during the transition into stationary phase (28, 29). However, in the mutant, high levels of *speB* transcription were observed at the late log phase of growth (Table 1), and these began to decline by the time transcription in the wild type was at peak levels (t = 2 h) (Table 1). This increased level of SpeB protein did not result in greater expression of protease activity in the Ffh$^-$ mutant, as SpeB-dependent cysteine protease activity in culture supernatant from the mutant was somewhat reduced from levels observed for the wild-type revertant (Table 1). This phenotype was medium dependent, as the supernatant fluids from the Ffh$^-$ mutant lacked both detectable cysteine protease activity (data not shown) and detectable levels of the SpeB polypeptide following culture in the glucose-deficient C medium, which is a medium that supports robust expression of SpeB by the wild-type revertant (Fig. 4). This defect was not due to a failure of the mutant to express SpeB, as the intracellular fraction of the mutant contained detectable levels of the SpeB polypeptide (Fig. 4, "cytoplasmic"). Glucose has a repressive effect on expression of SpeB (28, 29), and as expected, supplementing C medium with glucose resulted in repression of SpeB expression in the wild-type revertant (Fig. 4, compare WT_{rev} supernatant in "C medium + Glc" column to that in "C medium" column). However, the addition of glucose resulted in increased SpeB expression in the Ffh$^-$ mutant, as levels in the supernatant fraction for both the SpeB polypeptide (Fig. 4, "C medium + Glc") and cysteine protease activity (data not shown) were elevated to the levels observed in the wild-type revertant in unsupplemented C medium. Similarly to the effects on growth in C medium (see above), buffering to pH 6.5, but not the addition of NaCl, resulted in enhanced levels of SpeB in the supernatant fluids of the mutant (data not shown).

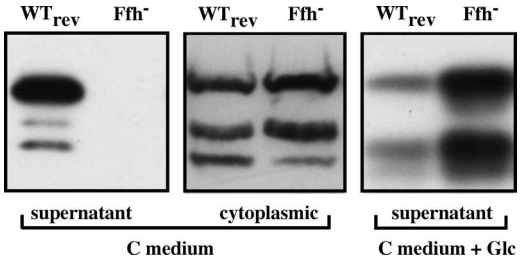

FIG. 4. Medium-dependent secretion defect. A Western blot analysis of SpeB expression from a mutant (Ffh$^-$) and a wild-type revertant (WT_{rev}) following culture in C medium or C medium supplemented with glucose (C medium + Glc) is shown. As indicated in the figure, both supernatant and cytoplasmic fractions were analyzed.

The SRP pathway is required for pathogenesis in a zebrafish model of necrotic myositis. Since the Ffh$^-$ mutant had both a medium-specific growth defect and a selective deficiency in its ability to export several toxins, it was of interest to determine whether the mutant was also altered in its ability to cause disease. The virulence of the Ffh$^-$ mutant was evaluated in a zebrafish model of necrotic myositis that reproduces several features commonly observed in streptococcal infection of human muscle, including local growth in muscle tissue, extensive necrosis of the infected tissue, and diminished inflammation (39). Infection of zebrafish with the wild-type revertant at approximately 10-fold the 50% lethal dose produced characteristic survival curves in which greater than 90% of infected zebrafish did not survive past day 3 postinfection (Fig. 5). Also, all fish infected with the wild-type revertant demonstrated a characteristic discolored lesion at the site of infection apparent 24 h postinfection that reflects the extent of the underlying necrosis in muscle (39), and all zebrafish that developed a lesion did not survive. In contrast, greater than 95% of zebrafish infected with the Ffh$^-$ mutant were still viable at day 3 (Fig. 5), demonstrating that the loss of the SRP rendered *S. pyogenes* significantly less virulent ($P < 0.0001$). Although lesions in zebrafish infected with the mutant were not as intense as or of equivalent area to those in zebrafish infected with the wild-type revertant, approximately 25% of the zebrafish infected with the mutant did develop lesions by day 2, and these animals typically survived and their lesions resolved. Lesion development had not been associated with any of the attenuated mutants analyzed previously (8, 39).

The SRP pathway is required for pathogenesis in a murine model of subcutaneous infection. Virulence of the Ffh$^-$ mutant was also examined in a murine model of subcutaneous infection, in which challenge with *S. pyogenes* HSC5 has been

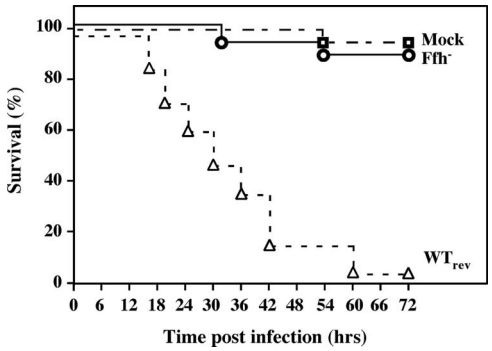

FIG. 5. The SRP is required for virulence in an animal model of necrotic myositis. Groups of zebrafish were challenged intramuscularly with 1×10^5 CFU of a mutant (Ffh$^-$) or a matched wild-type revertant (WT_{rev}) or were injected with sterile media alone (Mock). Survival was monitored for 72 h, and the data are presented as a Kaplan-Meier plot. Data are pooled from three independent experiments, each of which was conducted using 10 zebrafish per group. These data indicate that the mutant was significantly less lethal than the wild-type revertant ($P < 0.0001$).

observed to cause visible ulcer-like lesions following injection into subcutaneous tissue (8). Consistent with previous reports, all mice injected with the wild-type revertant had visible lesions by 48 h postinfection, while most mice infected with the Ffh$^-$ mutant failed to develop any evidence of a visible ulcer even following 72 h (Fig. 6) and 96 h (data not shown) of examination, demonstrating that the mutant was significantly less virulent ($P < 0.02$). A subset of mice infected with the mutant did develop ulcers (Fig. 6). However, the wild-type revertant caused lesions that were significantly larger in size than those caused by the mutant when compared at a time when lesion formation by the wild-type revertant had reached a maximum (72 h) ($P < 0.005$) (Fig. 6) and at subsequent time points over the duration of the experiment (96 h). The attenuation of the mutant is not due to its failure to secrete SLO and SPN, as mutants of this wild-type strain (HSC5) defective for expression of these two proteins are not attenuated in this model (J. Ghosh and M. G. Caparon, unpublished). These data provide additional evidence to suggest that Ffh plays an important role in *S. pyogenes* virulence.

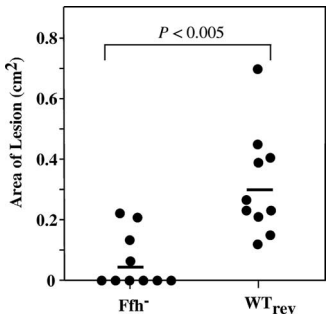

FIG. 6. The SRP is required for virulence in the murine subcutaneous ulcer model. Groups of female SKH1 hairless mice received a subcutaneous injection of 10^7 CFU of a mutant (Ffh$^-$) or a matched wild-type revertant (WT_{rev}) into the hind flank. Following 72 h, the area of any visible ulcer that formed at the site of injection was determined as described previously (8). Data shown are pooled from two independent experiments. Each symbol represents the area of the ulcer observed in an individual mouse, and the horizontal bar indicates the mean value obtained for the pooled set of 10 mice. As indicated in the figure, lesions caused by the WT_{rev} were significantly larger than those caused by the Ffh$^-$ mutant.

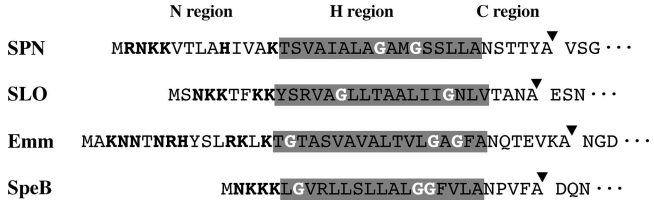

FIG. 7. Comparison of virulence factor signal sequences. The N, H, and C regions of the signal peptides of SPN (SPy_0165), SLO (SPy_0167), Emm1 (SPy_2018), and SpeB (SPy_2038) are shown. Positively charged residues in the N region and the residues of the hydrophobic H region are indicated in bold type and by gray highlighting, respectively. Helix-disrupting glycine residues in the H regions are indicated in white type. The signal peptidase cleavage site (indicated by the arrowhead) predicted computationally by SignalP, version 3.0 (6), agreed with the mature N termini that have been determined experimentally and reported for each protein.

DISCUSSION

These data suggest that the nonessentiality of the SRP pathway may be a general feature of the streptococci. However, the SRP does contribute to a number of important functions of *S. pyogenes*, including the ability to efficiently utilize noncarbohydrate substrates for growth, to secrete several proteins involved in host cell-pathogen interactions, and to cause disease in multiple animal models of streptococcal infection. The fact that the SRP is not required for growth should allow more refined molecular studies of this important pathway for targeting presecretory proteins that should provide insight into the pathway in other bacterial species where the SRP is essential. Further analyses of the SRP pathway in *S. pyogenes* should also be useful for understanding how targeting pathways may influence the trafficking fate of secreted proteins during infection.

While it is clear that the signal sequence is important for recognition by the SRP, the specific features of the signal sequence that dictate whether the nascent presecretory protein is routed to the SRP pathway or to another pathway are not clear. Signal sequences themselves canonically have three regions, including an amino-terminal region enriched in positively charged residues (N region), a central region consisting of hydrophobic residues (H region), and a moderately polar carboxy-terminal domain (C region) (Fig. 7). Studies of *E. coli* have suggested that while SRP is not an exclusive signal, the probability of SRP recognition of any one signal peptide does correlate with its degree of hydrophobicity (26, 27). Preliminary results from our analysis using the computational method of Huber et al. (26) failed to identify any correspondence between hydrophobicity and whether the signal sequences of the four proteins analyzed in this study were routed to the SRP pathway. A complication is that the longer hydrophobic regions typical of a gram-positive signal sequence generally render them quite hydrophobic overall. The signal sequences of SLO, SPN, M protein, and SpeB are unremarkable in this regard and have H regions encompassing 17 to 18 residues

(Fig. 7). It has also been suggested that the presence of glycine residues in the H region can destabilize formation of an alpha helix that results in an inability to interact with the SRP (1). An examination of the four streptococcal signal sequences shows that their H regions all contain multiple glycine residues (Fig. 7). Thus, there are no obvious characteristics of these signal sequences that distinguish between their routing pathways.

Evidence suggests that an important function of the SRP pathway is in the insertion of integral membrane proteins (44). Since nutrient transporters typically include an integral membrane component, the loss of a transporter could explain the medium-specific growth defect of the *S. pyogenes* Ffh$^-$ mutant. As the growth and SpeB secretion defect could be reversed by the addition of glucose, it is possible that an important peptide transporter requires the SRP for its insertion into the membrane, making the mutant more dependent on carbohydrates for growth and the secretion of virulence factors. A differential dependence of nutrient transporters on the SRP pathway may also explain recent studies that showed that depletion of the SRP in *B. subtilis* resulted in a pronounced secretion defect when cells were grown in minimal media that was much less severe when cell were grown under rich-medium conditions (49). Likewise, the viable SRP null mutants of *S. mutans* are typically grown in complex rich media (23). An increased requirement for glucose could help to explain the *S. pyogenes* Ffh$^-$ mutant's attenuated ability to cause disease. Analysis of the transcriptome during infection has shown that *S. pyogenes* experiences a significant starvation for carbohydrates in well-developed necrotic lesions (29). This idea is supported by evidence suggesting that the nutritional environment experienced by *S. pyogenes* in a necrotic lesion is similar to the nutritional landscape presented by unsupplemented C medium during growth in culture (29) and that this medium had the most restrictive effect on the Ffh$^-$ mutant's growth and secretion of the several media evaluated.

Alternatively, the Ffh$^-$ mutant's requirement for glucose for growth and for secretion of SpeB in C medium may reflect the complex transcriptional regulatory networks that control expression of SpeB. It is known that transcription of the gene that encodes this protease is highly regulated and sensitive to several different regulatory pathways, including those that respond to nutritional cues (29). The observation that temporal regulation of *speB* transcription is altered in the mutant may suggest that it lacks a transporter for a metabolite that is sensed by the SpeB regulatory network for control of temporal expression. Alternatively, it may lack an as-yet-unknown membrane-associated sensory protein involved in regulation. Also, while the mutant grew poorly in unsupplemented C medium, it did express SpeB, although it failed to secrete the protease. A failure to secrete the protease could be a factor that contributes to poor growth in unsupplemented medium. It seems unlikely that this could be the sole reason for the growth defect, in that conditions which promote *speB* transcription (low pH) rescued growth and yet a condition that represses *speB* transcription (high salt) did not. The ability of glucose to rescue growth was associated with a restored ability to secrete SpeB. Since under these conditions secretion of SpeB itself did not require Ffh, it is possible that there is an accessory secretion factor required for high-level secretion that is itself regulated by glucose and dependent on Ffh for insertion into the membrane. Accessory factors that promote high levels of secretion have been described for other gram-positive bacterial species (for a review, see reference 45).

Analysis of the trafficking of thioredoxin in *E. coli* has suggested that proteins exported by SRP-dependent signal sequences are those that have the ability to fold rapidly in the cytoplasm (26). If allowed to accumulate in the cytoplasm for any length of time, it is likely that these proteins would jam the translocons if an attempt was made to secrete them in a partially folded state, a situation that can be bypassed by cotranslational secretion (26). Support for this function of the SRP pathway comes from the observation that SPN depends on the SRP for secretion. As an NAD glycohydrolase, SPN is indiscriminate in its source of substrate and is toxic to both streptococcal and host cells. In fact, the ability of *S. pyogenes* to produce SPN is absolutely dependent on coexpression of an immunity factor known as immunity factor for SPN (36). This immunity factor acts as a competitive inhibitor of SPN′s NAD substrate to ameliorate its toxicity (36). Interestingly, immunity factor for SPN resides exclusively in the streptococcal cytoplasm, suggesting that even if a few molecules of SPN go off-pathway and fold rapidly in the cytoplasm, the result is an inability to sustain viability. Secretory stress induced by partially folded proteins could also explain the fact that, even under conditions where it was growing robustly, the mutant appeared to be experiencing a stress response, as evidenced by the enhanced association of DnaK and GroEL with membranes. A similar stress response has been observed to occur in Ffh-depleted *B. subtilis* strains (49) and in *S. mutans* mutants (24).

Our analysis has contributed to the emerging picture that protein secretion by *S. pyogenes* and other gram-positive bacteria is a complex and organized process. For example, *S. pyogenes* has been shown to have a distinct membrane microdomain dedicated to protein secretion known as the Ex-Portal (42), which may serve as a protein-folding organelle by promoting the interaction of unfolded nascent secretory proteins with accessory maturation factors (43). Secreted proteins that are substrates of the sortase pathway are not distributed randomly in the cell wall. Rather, the M protein first appears linked to the cell wall predominantly along the septum, while protein F first appears at higher concentrations at the cell poles (11). These events imply that there is considerable cooperation between the protein export and cell wall synthesis machineries and that intracellular routing signals play an important role in dictating trafficking fate. This notion is supported by the observation that exchange of signal sequences between M protein and protein F alters how the proteins appear in the wall (11). Thus, while the SRP appears dispensable for growth of various species of streptococci, understanding how the SRP pathway contributes to the secretion process is essential for understanding how protein secretion contributes to the pathogenic mechanisms by which this group of bacteria causes disease.

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