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The Majority of 9,729 Group A Streptococcus Strains Causing Disease Secrete SpeB Cysteine Protease: Pathogenesis Implications

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Group A streptococcus (GAS), the causative agent of pharyngitis and necrotizing fasciitis, secretes the potent cysteine protease SpeB. Several lines of evidence suggest that SpeB is an important virulence factor. SpeB is expressed in human infections, protects mice from lethal challenge when used as a vaccine, and contributes significantly to tissue destruction and dissemination in animal models. However, recent descriptions of mutations in genes implicated in SpeB production have led to the idea that GAS may be under selective pressure to decrease secreted SpeB protease activity during infection. Thus, two divergent hypotheses have been proposed. One postulates that SpeB is a key contributor to pathogenesis; the other, that GAS is under selection to decrease SpeB during infection. In order to distinguish between these alternative hypotheses, we performed casein hydrolysis assays to measure the SpeB protease activity secreted by 6,775 GAS strains recovered from infected humans. The results demonstrated that 84.3% of the strains have a wild-type SpeB protease phenotype. The availability of whole-genome sequence data allowed us to determine the relative frequencies of mutations in genes implicated in SpeB production. The most abundantly mutated genes were direct transcription regulators. We also sequenced the genomes of 2,954 GAS isolates recovered from nonhuman primates with experimental necrotizing fasciitis. No mutations that would result in a SpeB-deficient phenotype were identified. Taken together, these data unambiguously demonstrate that the great majority of GAS strains recovered from infected humans secrete wild-type levels of SpeB protease activity. Our data confirm the important role of SpeB in GAS pathogenesis and help end a long-standing controversy.

Bacterial pathogens often secrete proteases that are important virulence factors (1). Proteases participate in many aspects of pathogen-host interaction, including nutrient acquisition, modification of other bacterial proteins, inactivation of host immune molecules, and tissue destruction (1). Thus, protease virulence factors are essential for the pathogen to colonize mucosal surfaces, evade the host immune response, invade deep soft tissue, disseminate to distant anatomic sites, and spread to new hosts (1). These features make proteases relevant targets for basic research and translational projects geared to creating new diagnostics and vaccines.

Group A streptococcus (GAS), a human-specific pathogen, is an important cause of morbidity and mortality worldwide (2). The pathogen is estimated to cause 600 million infections annually, including 10,000 to 15,000 severe invasive infections in the United States (2). Infections range in severity from self-limiting pharyngitis ("strep throat") to life-threatening necrotizing fasciitis ("flesh-eating disease") (3). GAS pathogenesis is mediated by many secreted and cell wall-associated toxins, superantigens, and virulence factors (3). Streptococcal pyrogenic exotoxin B (SpeB), a potent broad-spectrum cysteine protease secreted by GAS, is among the more extensively studied bacterial proteases (4, 5). The chromosomally encoded *speB* gene is present in virtually all GAS strains (6). Several lines of study have provided strong evidence that SpeB is an important GAS virulence factor (4). SpeB protease can modify many GAS proteins, such as the antiphagocytic M protein, the SmeZ superantigen, and the cytolytic toxin streptolysin O (SLO) (7, 8), cleave the host immune molecules pre-IL-1β (pre-interleukin-1β) and complement component C3b (9), induce the apoptosis of host macrophages and epithelial cells (10), degrade the extracellular matrix proteins fibronectin and vitronectin (11), and activate host matrix metalloproteases responsible for tissue repair (12).

The generation of enzymatically active SpeB by GAS is a very complex multistage process that involves at least 21 gene products (4) (Table 1). *speB* is transcribed from two promoters and is directly regulated by three transcription factors (13). Transcription and posttranscriptional processing are regulated by various environmental signals, such as changes in pH or electrolytes and expression of other GAS-derived molecules (14, 15). The SpeB zymogen is secreted through an organelle known as the GAS ExPortal (16) before undergoing a series of intra- and intermolecular processing steps to generate the enzymatically active mature SpeB protease (17, 18). Each stage is regulated by multiple GAS accessory factors (Table 1). Disruption of any of these components may render a GAS strain deficient in secreted SpeB protease activity (19–24).

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TABLE 1 Genes implicated in altered secreted SpeB protease activity

Category and gene	Function	Effect of polymorphisms
Direct transcriptional regulators		
ropB	Transcription factor	Decreased transcription of *speB*
covRS	Two-component system	Decreased transcription of *speB*
ccpA	Transcription factor	Decreased transcription of *speB*
Indirect regulators and posttranscriptional processing		
clpX	ATP-dependent subunit of Clp protease	Decreased transcription and processing
fabT	Transcription factor	Decreased transcription and processing
luxS	Transcription factor	Decreased transcription and processing
pel	Unknown	Decreased transcription and processing
sagP	Transcription factor	Decreased transcription and processing
Secretion and posttranslational processing		
ftsH	Membrane-associated protease	Decreased maturation of SpeBz to SpeBm
gdpP	c-di-AMP phosphodiesterase	Decreased maturation of SpeBz to SpeBm
htrA	Periplasm-associated protease	Decreased maturation of SpeBz to SpeBm
mtsR	Transcription factor, regulator of *prsA*	Decreased maturation of SpeBz to SpeBm
prsA	Peptidyl-prolyl *cis-trans* isomerase	Decreased maturation of SpeBz to SpeBm
ropA	Peptidyl-prolyl *cis-trans* isomerase	Decreased maturation of SpeBz to SpeBm
Growth phase and environmental signaling		
codY	Transcription factor, growth phase regulation	Increased transcription of *speB*
lacD.1	Transcription factor, environmental stimuli	Increased transcription of *speB*
nra	Transcription factor, indirect regulator of *speB*	Increased transcription of *speB*
svr	Transcription factor, growth phase regulation	Increased transcription of *speB*
vfr	Signal peptide for *ropB* activation	Increased transcription of *speB*

An epidemic serotype *emm1* GAS clone has been described and extensively studied because it is a major cause of severe invasive human infections worldwide (25–30). As a consequence of several studies, the idea has arisen that GAS may be under selective pressure to decrease secreted SpeB protease activity during infection (31–33). This decrease has been linked to the initiation of invasive infection, increased disease severity, and a poor outcome on the basis of analysis of convenience samples rather than comprehensive population-based strain samples (23). However, other studies performed by multiple investigators have reported that the secreted SpeB protease is a key virulence factor contributing to tissue destruction, dissemination, and mortality in invertebrate, mouse, and nonhuman primate models (9, 22, 34–41). Moreover, SpeB is expressed in humans with invasive disease (42–44); infected humans generate anti-SpeB antibodies (43–45); and immunization of mice with SpeB or SpeB-derived protein fragments protects against lethal challenge (36, 46–48). Mice treated with a protease inhibitor that inactivates SpeB are similarly protected against invasive disease (49). In this regard, Eriksson et al. reported that acute-phase sera obtained from patients with streptococcal toxic shock syndrome (a severe life-threatening infection) had a significantly lower capacity to neutralize SpeB activity than sera from patients with uncomplicated bacteremia or erysipelas (43). Similarly, acute-phase sera from patients with GAS bacteremia had a significantly lower SpeB-neutralizing ability than sera from patients with uncomplicated tonsillitis (44).

Thus, two divergent ideas have emerged about the role of SpeB in GAS pathogenesis. One idea postulates that SpeB protease is a key contributor to pathogen-host interaction in several phases of pathogenesis. If so, the great majority of strains cultured from diseased humans would be expected to retain the capacity to produce wild-type levels of secreted SpeB protease. Alternatively, if the other idea, that decreased SpeB production is a key driver of invasive infections (31–33), is correct, then the majority of strains cultured from infected humans should lack SpeB protease activity as a consequence of chromosomal mutations in one or more of the 21 genes implicated in SpeB production (Table 1). It is important to resolve this matter, because it has implications for our understanding of GAS pathogenesis and may influence the direction of translational research efforts such as vaccine development.

We recently sequenced the genomes of many thousands of GAS strains recovered from infected humans, focusing on organisms recovered in prospective comprehensive population-based studies conducted in many different parts of the world (27, 50–52). These studies permit us to contribute information that may help in evaluating the two ideas bearing on SpeB production and human infections. We measured the secreted SpeB protease activities made by 6,775 *emm1*, *emm89*, *emm59*, and *emm28* GAS strains. These serotypes were selected because they are among the most common causes of severe invasive disease worldwide (2, 53). The results demonstrated that the great majority (84.3%) of GAS strains retain wild-type secreted SpeB protease activity, regardless of the disease type or strain serotype. Similarly, whole-genome sequence analysis of 2,975 *emm1* GAS strains recovered in a nonhuman primate model of necrotizing fasciitis found that none had a mutation that would lead to a SpeB-deficient phenotype. We interpret these data as strong evidence for an important role of SpeB in GAS pathogenesis, consistent with findings from many

human clinical studies and animal infection models using isogenic mutant strains.

MATERIALS AND METHODS

Serotype *emm1* strains. We studied 3,615 *emm1* GAS strains collected over 4 decades from patients in nine distinct geographic locations in North America and Europe (27). This strain sample was recently described in detail, including whole-genome sequence analysis (27). Most strains were collected as part of comprehensive prospective population-based surveillance studies. Invasive strains included 346 from Ontario, Canada (1997 to 2009), 436 from Denmark (1973 to 2013), 155 from East Germany (1969 to 1991), 509 from Finland (1988 to 2011), 50 from Iceland (1988 to 2011), 215 from Norway (1997 to 2009), 482 from Sweden (1996 to 2012), 340 from Georgia, United States (1995 to 2010), and 474 from Minnesota, United States (1995 to 2010). In addition, 11 strains of historic interest from other countries and times were included (27). Strains from Georgia (Atlanta metropolitan area) and Minnesota (statewide) were collected as part of the Active Bacterial Core Surveillance Program administered by the U.S. Centers for Disease Control and Prevention (53). Pharyngitis strains included 597 GAS strains from Finland (1988 to 1997), most of which were recovered in a population-based surveillance study during years overlapping with those of the Finnish invasive-strain study (54).

Serotype *emm89* strains. The 1,181 *emm89* strains were collected as part of comprehensive prospective population-based surveillance studies conducted in three countries. These strains were recovered from patients with invasive infections in Finland ($n = 286$; years, 2003 to 2014), Iceland ($n = 24$; years, 1997 to 2008) and 10 states in the United States ($n = 870$; years, 1995 to 2013), and have recently been described in detail (52). The *emm89* reference strains MGAS11027 and MGAS23530 have been sequenced to closure (52).

Serotype *emm59* strains. The 704 GAS *emm59* invasive strains were collected as part of comprehensive prospective population-based surveillance studies conducted in the United States ($n = 50$) and Canada ($n = 638$) or had historic interest ($n = 16$) (50, 51, 53, 55–57).

Serotype *emm28* strains. The 1,275 serotype *emm28* invasive strains were collected as part of comprehensive prospective population-based surveillance studies conducted in Finland ($n = 48$), Iceland ($n = 27$), Ontario, Canada ($n = 204$), and the United States ($n = 996$).

SpeB secreted protease activity assay. SpeB protease activity secreted by the 5,775 GAS strains was evaluated with a standard casein hydrolysis (milk plate) assay (34). Briefly, strains were grown from cryopreserved stocks for 12 to 24 h on tryptic soy agar (TSA) supplemented with 5% sheep blood (Becton Dickinson) at 37°C under 5% CO_2. Each strain was then subcultured for 16 h to the early-stationary growth phase in Todd-Hewitt medium supplemented with 0.2% yeast extract (Difco) and was inoculated in triplicate by stabbing TSA plates supplemented with 5% skim milk (Teknova) with a solid-bore plastic needle (Globe Scientific). The plates were incubated for 24 h at 37°C in an anaerobic chamber (Mitsubishi Gas Chemical Co.). Secreted SpeB protease activity was determined by measuring the zone of caseinolysis around each inoculation site using a digital caliper (Fisher Scientific). The mean zone size was interpreted relative to those of the SpeB wild-type *emm1* strain MGAS2221 (58), the SpeB-deficient *emm1* strain MGAS5005, which has an inactivating mutation in the gene encoding CovS (59), and the isogenic mutant SpeB-negative strain MGAS5005Δ*speB*, which has the gene encoding SpeB deleted.

Gene sequencing and polymorphism discovery. The whole-genome sequence data and the bioinformatic analysis strategy used for the 3,615 *emm1* strains have been reported previously (27). Genomewide polymorphisms (single nucleotide polymorphisms [SNPs] and insertions and deletions [indels]) were first identified relative to the very high quality genome of the *emm1* reference strain MGAS5005 (GenBank accession number CP000017) with VAAL software (60). The nature of SNPs (coding/noncoding, synonymous/nonsynonymous, etc.) was determined with the Perl script SNPeffect-0.2.pl. An R script was used to export a comma-separated values (CSV) file containing all polymorphisms identified in the 21 genes implicated in altered secreted SpeB protease activity (27). The complete upstream noncoding regions and coding regions of the 21 genes were evaluated for every strain identified as having a SpeB-deficient phenotype (Table 1). Synonymous single nucleotide polymorphisms (coding for the same amino acid) and polymorphisms identified in multiple strains with a wild-type SpeB phenotype (not SpeB altering) were excluded from the analysis. For SpeB-deficient strains initially found by this analysis strategy to lack a gene polymorphism that could account for the protease-negative phenotype, the whole-genome sequence data were reanalyzed with a second bioinformatics strategy using Trimmomatic, Musket, SMALT, and FreeBayes as described previously (56). FreeBayes (61) is better able than VAAL to identify minority subpopulations that could contribute to an altered SpeB protease phenotype. All polymorphisms contributing to a SpeB-deficient phenotype were confirmed by visualization of the mapped reads using Tablet (62). Previously published whole-genome sequence data for SpeB-deficient *emm89*, *emm59*, and *emm28* strains were analyzed using the same bioinformatics process.

Experimental animal infections. A nonhuman primate model of necrotizing fasciitis was used as described previously (27, 39, 63). Four adult cynomolgus macaques (*Macaca fascicularis*) (Charles River BRF) were sedated with ketamine, shaved over the upper posterior thorax, and premedicated with a 25-μg/h fentanyl patch that was sutured in place over the shaved area (Sandoz, Inc.). A mesh jacket (Lomir Biomedical, Inc.) was fitted to each animal to protect the fentanyl patch. Each animal was inoculated intramuscularly in the anterior thigh at a uniform depth with 1×10^8 CFU/kg of serotype *emm1* strain MGAS2221 in 200 μl phosphate-buffered saline (PBS). Strain MGAS2221 was selected because it has a wild-type allele (i.e., the most common allele) for all major GAS transcriptional regulators, including *covRS* and *ropB*, is genetically representative of contemporary epidemic serotype M1 strains, and has been used in numerous animal experiments (27). The inoculation site was marked with a tattoo. The animals were observed continuously, sacrificed at 24 h post-inoculation, and necropsied. At necropsy, the quadriceps muscle was removed *en bloc*, serially sectioned in 0.5-cm slices, and inspected visually. Biopsy specimens of grossly purulent tissue (approximately 0.5 g each) were collected from the inoculation site, 1 cm from the caudal margin, and 1 cm from the cephalic margin. Each muscle sample was homogenized (Omni International), serially diluted in sterile PBS, and plated in quadruplicate on Trypticase soy agar supplemented with 5% sheep blood (Becton Dickinson and Company). Following incubation at 37°C under 5% CO_2 for 18 h, the plates were removed and were visually inspected in order to enumerate colonies and confirm the absence of contaminating organisms. In total, 2,954 GAS colonies (range, 698 to 760 per animal; approximately one-third each from the inoculation site, caudal margin, and cephalic margin) were selected for whole-genome sequencing as described previously (27). To avoid introducing bias due to the colony phenotype, every colony was taken from plates prepared from the dilution that produced approximately 100 to 200 colonies/plate. The study protocol was approved by the Institutional Animal Care and Use Committee at the Houston Methodist Research Institute.

RESULTS

Secreted SpeB protease activity among 3,615 *emm1* strains. A casein milk plate hydrolysis assay was used to test the hypothesis that most *emm1* GAS strains have a wild-type SpeB proteolytic phenotype. Serotype *emm1* strains were selected for study because they are a frequent source of human pharyngeal and sterile-site infections (2), display epidemic behavior (2), and have caused marked increases in the frequency and severity of invasive infections beginning in the mid- to late 1980s (25–30). The 3,615 strains studied were from comprehensive population-based studies conducted in 9 disparate geographic locations over approximately 40 years (27). The majority of these strains were cultured

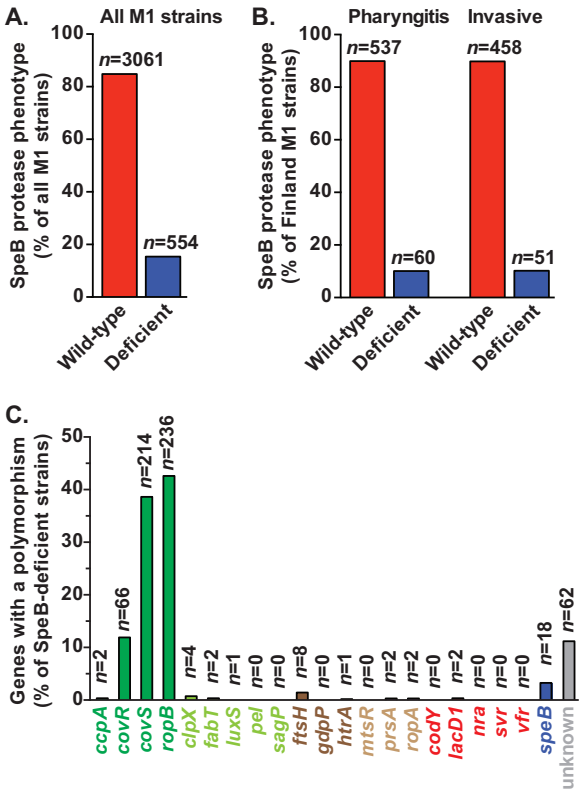

FIG 1 A majority of *emm1* GAS strains have a wild-type secreted SpeB protease phenotype. (A) Casein milk plate hydrolysis assays were performed on 3,615 *emm1* GAS strains collected in comprehensive population-based studies conducted at 9 geographically disparate sites in North America and Europe. The number of strains with a SpeB-wild-type (red bar) or deficient (blue bar) phenotype is shown. (B) Comparison of secreted SpeB protease activities in strains recovered within overlapping time intervals from patients in Finland with pharyngeal or invasive infections. (C) Whole-genome sequence data were analyzed in order to identify polymorphisms in 21 genes encoding products implicated in secreted SpeB protease activity, including those involved in direct transcriptional regulation (dark green), indirect transcriptional regulation or posttranscriptional processing (light green), secretion (dark brown), posttranslational processing (light brown), or growth phase or environmental signaling (red), as well as *speB* and its upstream regulatory region (blue). In some SpeB-deficient strains, no likely causative polymorphisms were identified (gray).

from patients with invasive infections. In agreement with our hypothesis, 3,061 strains (84.6%) had wild-type secreted SpeB protease activity (Fig. 1A).

A small case series had previously suggested that severe invasive infections were associated with a SpeB-deficient phenotype (23). We compared the SpeB phenotype results for GAS strains recovered from patients with invasive or pharyngeal infections in Finland within overlapping time intervals (27). No significant difference in secreted SpeB protease activity was found between these two patient groups (Fig. 1B) (*P*, not significant by the chi-square test).

Gene polymorphisms associated with decreased secreted SpeB protease activity among *emm1* strains. Twenty-one genes have been implicated in a complicated process that ultimately generates enzymatically active SpeB (Table 1) (4). To determine the relative frequency of mutations associated with decreased secreted SpeB protease activity, we analyzed whole-genome sequence data for the 554 SpeB-deficient *emm1* GAS strains we identified (27). The three most frequently mutated genes encode proteins that are known to directly regulate *speB* transcription (Fig. 1C). These genes encode the two-component system CovRS (control of virulence regulator/sensor) and RopB (regulator of protease B). A total of 66 (11.9%) SpeB-deficient *emm1* strains have mutations in *covR*, 214 (38.6%) have mutations in *covS*, and 236 (42.6%) have mutations in *ropB*. Polymorphisms in the other genes implicated were infrequently identified (range, 0 to 8 polymorphisms per gene [0 to 1.4%]). Only 18 strains (3.2% of SpeB-deficient strains) had a polymorphism in the *speB* coding region or the upstream promoter region. Of note, no polymorphism that likely explains the SpeB-deficient phenotype was identified for 62 strains (11.2% of SpeB-deficient *emm1* strains) (Fig. 1C).

Secreted SpeB protease activity among 1,181 *emm89* strains. To further test our hypothesis that most GAS strains recovered from infected humans with invasive infections have a wild-type SpeB production level, we examined the secreted protease activity in 1,181 *emm89* strains for which we also have full-genome sequence data (52). Serotype *emm89* strains were analyzed because they are a frequent cause of human infections (2, 53, 64–66). The *emm89* strains analyzed were collected in comprehensive population-based studies conducted in Finland, Iceland, and the United States. Like our findings for the *emm1* strains, the milk plate hydrolysis data showed that the great majority of *emm89* GAS strains ($n = 936$ [79.3%]) had a wild-type secreted SpeB protease phenotype (Fig. 2A). Among the 245 *emm89* strains deficient in SpeB activity, frequently mutated genes included *covR* (mutated in 37 strains [15.1% of SpeB-deficient *emm89* strains]), *covS* (100 strains [40.8%]), and *ropB* (96 strains [39.2%]) (Fig. 2B).

Secreted SpeB protease activity among 704 *emm59* strains. We next assessed secreted SpeB protease activity among 704 *emm59* strains that have been characterized by whole-genome sequencing (50, 51, 55, 56, 67). Most of these *emm59* organisms were recovered as part of comprehensive population-based epidemiologic studies conducted during a countrywide epidemic of severe invasive infections in Canada (57) or were cultured from patients with invasive infections in the United States (53). The results were closely similar to our findings for the *emm1* and *emm89* strains: the great majority of the *emm59* GAS strains ($n = 631$ [89.6%]) produced wild-type levels of secreted SpeB protease activity (Fig. 2C). The most frequently mutated genes among the protease-deficient *emm59* organisms were *covR* (mutated in 15 strains [20.5% of SpeB-deficient *emm59* strains]), *covS* (23 strains [31.5%]), and *ropB* (24 strains [32.9%]) (Fig. 2D).

Secreted SpeB protease activity among 1,275 *emm28* GAS strains. We next measured SpeB protease activity secreted by 1,275 *emm28* strains. Most of these *emm28* GAS organisms were recovered as part of comprehensive population-based epidemiologic studies conducted in Finland, Iceland, Ontario (Canada), and the United States. Serotype *emm28* GAS strains are a numerically prominent cause of human infections, particularly life-threatening puerperal sepsis (53, 68). As observed for strains of the other GAS *emm* types studied, the great majority of *emm28* strains ($n = 1,083$ [84.9%]) had wild-type secreted SpeB protease activity (Fig. 2E). Although not all the genomes of the *emm28* GAS strains in these collections have been sequenced yet, data were available for 49 SpeB-deficient organisms. Mutations in *covR* (mutated in 2 strains [4.1% of SpeB-deficient *emm28* strains for which whole-genome sequence data were available]), *covS* (11 strains [22.4%]),

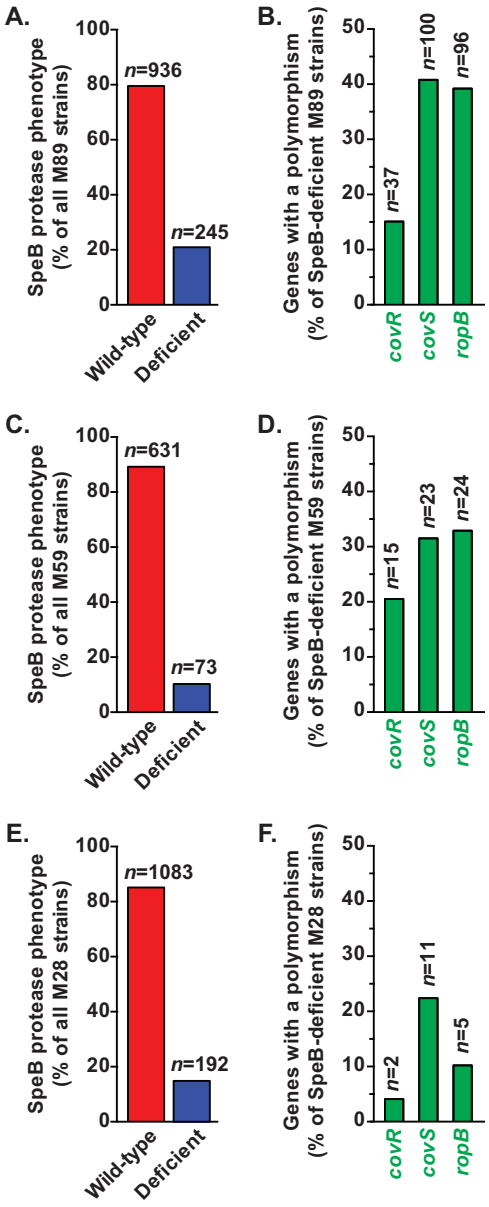

FIG 2 The majority of *emm89*, *emm59*, and *emm28* strains have a wild-type secreted SpeB protease phenotype. Casein milk plate hydrolysis assays were performed on 1,181 serotype *emm89* (A), 704 *emm59* (C), and 1,275 *emm28* (E) GAS strains collected in comprehensive population-based studies conducted at geographically distinct sites in North America and Europe. The numbers of strains with a SpeB-wild-type (red bars) or deficient (blue bars) phenotype are shown. Whole-genome sequence data were analyzed to identify polymorphisms in *covR*, *covS*, and *ropB* in SpeB-deficient *emm89* (B), *emm59* (D), and *emm28* (F) strains.

and *ropB* (5 strains [10.2%]) occurred at rates similar to those found in the *emm1*, *emm89*, and *emm59* strains (Fig. 2F).

Whole-genome sequence analysis of 2,954 serotype *emm1* GAS strains recovered in a nonhuman primate model of necrotizing fasciitis. When mice are experimentally infected with wild-type GAS, SpeB-deficient mutants (most commonly due to mutations in *covRS*) arise rapidly and may increase to numerical prominence (19, 33, 58). However, our data clearly demonstrate that the great majority of GAS strains recovered from humans with pharyngitis or invasive infections retain a wild-type SpeB phenotype (Fig. 1 and 2). To further test the hypothesis that most GAS strains recovered in invasive infections secrete SpeB protease, we sequenced the genomes of 2,954 strains recovered from 4 nonhuman primates (the most human-relevant animal model possible) with experimental necrotizing fasciitis caused by a wild-type *emm1* GAS strain. In agreement with our hypothesis, none of these strains acquired a mutation in any of the 21 genes known to be needed for GAS to secrete wild-type SpeB protease activity.

DISCUSSION

The progeny of a globally disseminated serotype *emm1* GAS clone that arose in the early 1980s are responsible for an ongoing epidemic of severe invasive infections (25, 26). Due to its human health importance, the *emm1* epidemic clone has been extensively studied by many investigators (25–30). The identification of polymorphisms in several genes that confer a SpeB-deficient phenotype has led, in part, to the hypothesis that epidemic *emm1* GAS strains are under intense selective pressure to decrease secreted SpeB protease activity during infection (19–24). Reinforcing this idea, Kansal et al., using a small sample of strains, reported an inverse relationship between SpeB proteolytic activity and disease severity (23). This report has been repeatedly cited to support the idea that SpeB-deficient strains are driving the *emm1* epidemic. However, as shown in the present study, data from 3,615 *emm1* GAS strains recovered in comprehensive population-based studies at nine geographically distinct sites on two continents do not support this hypothesis. Our data clearly demonstrate that a SpeB-deficient phenotype is very uncommon among epidemic *emm1* organisms (Fig. 1). Moreover, the SpeB-deficient phenotype occurs at the same frequency among strains causing invasive infections and those causing pharyngitis (Fig. 1). Thus, the only reasonable conclusion is that strains with genetic changes resulting in decreased secreted SpeB protease activity are not major contributors to the global epidemic of *emm1* GAS infections. Analysis of 1,181 *emm89*, 704 *emm59*, and 1,275 *emm28* strains produced essentially identical findings (Fig. 2). That is, collectively among 6,775 strains taken from comprehensive population-based collections of 4 different GAS *emm* types, only 15.7% of strains have a SpeB-deficient phenotype.

Our data have important implications for understanding the emergence of SpeB-deficient strains of *emm1* GAS and presumably other *emm* types of GAS. We and other investigators have described polymorphisms in the two-component signal transduction system *covRS* that markedly alter the transcriptome profile of GAS, including decreasing *speB* expression (31, 58, 69). Mutations in *covRS* are readily generated by passage of wild-type strains through mice to create progeny with an "invasive phenotype" (58). One school of thought has been that elimination of secreted SpeB protease activity, which may degrade other GAS virulence factors expressed in the SpeB wild-type condition, acts as the molecular switch to increased virulence of the epidemic clone during invasive infection (31, 32, 58). However, although loss of SpeB proteolytic activity occurs in experimentally infected mice and some humans, our casein hydrolysis and genomic data rule out the speculative idea that this phenotype is the primary driver of the epidemic GAS disease. If GAS were under selection to develop a SpeB-deficient phenotype in infected humans, then mutations that eliminate secreted SpeB proteolytic activity would be identified at a far higher frequency. Moreover, mutations in *speB* itself,

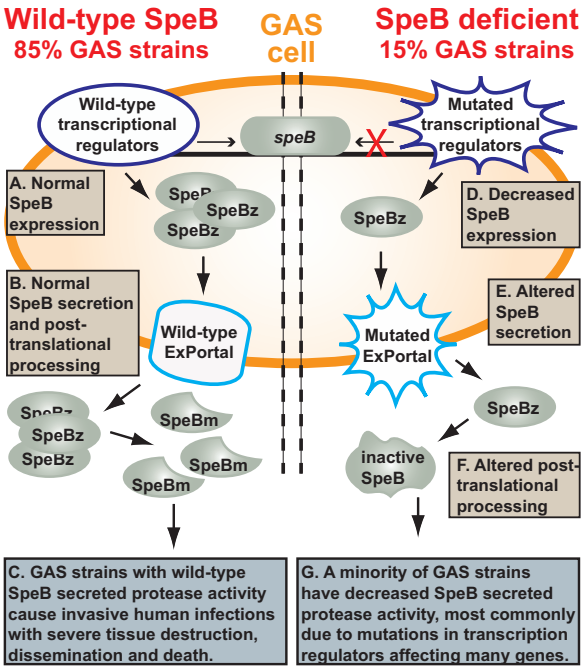

FIG 3 Model summarizing secreted SpeB protease activity. (Left) In wild-type strains (approximately 85% of GAS strains cultured from infected humans), normal amounts of SpeB transcript are made (A), the enzymatically inactive zymogen (SpeBz) is translated and secreted through the ExPortal (B), and SpeBz is processed through multiple intra- and intermolecular steps to yield the potent broad-spectrum mature cysteine protease (SpeBm), which is a key virulence factor for human disease (C). (Right) A minority of GAS strains (15%) have a SpeB-deficient phenotype caused by mutations in genes implicated in transcription or posttranscriptional regulation (D), secretion (E), or posttranslational processing (F). SpeB-deficient strains constitute a minority among organisms causing either human invasive infections or pharyngitis (G).

rather than in transcription factors that regulate many other GAS virulence factors, would be expected to occur more frequently. Our data show that only genes encoding direct regulators of *speB* expression (*covRS* and *ropB*) accumulated more polymorphisms than would be expected to occur by chance alone (Fig. 1 and 2) (27). Furthermore, 28 SpeB-deficient *emm1* strains had polymorphisms in both *covRS* and a second gene that would confer decreased secreted SpeB protease activity. We also identified 19 *emm89* and 6 *emm59* GAS strains with polymorphisms in both *covRS* and *ropB*. That is, *covRS* and *ropB* mutations may be occurring in strains that already have a SpeB-deficient phenotype, or vice versa. Two independent studies have similarly described *covRS* mutations arising in SpeB-deficient strains with preexisting *ropB* mutations (70, 71). In addition, we identified 8 *covRS*-mutated SpeB-deficient *emm1* strains that were recovered in cases of human pharyngitis, demonstrating that these mutations are not limited to invasive infections. Taken together, our data strongly support an alternative model in which the great majority of *emm1* (and *emm89*, *emm59*, and *emm28*) GAS strains causing human infections retain a wild-type SpeB phenotype (Fig. 3). Less commonly, GAS may acquire polymorphisms in major transcription regulators, such as *covRS* or *ropB*, that influence the expression of a large number of virulence genes. Thus, selection pressure to eliminate secreted SpeB protease activity may not be a primary factor; rather, GAS may be under selection to increase the expression of genes encoding other potent virulence factors regulated by *covRS* (58), including the antiphagocytic hyaluronic acid capsule (63), Sda1 DNase (72), and the cytolytic toxins streptolysin O (SLO) and NAD^+ glycohydrolase (SPN) (52). In this regard, we recently demonstrated that upregulation of SLO and SPN exotoxin production by recombinational evolution was the critical molecular trigger igniting intercontinental epidemics of *emm1* and *emm89* GAS that have affected tens of millions of people (27, 52).

The relationship between SpeB protease production and disease pathogenesis is a complicated area of investigation that has been studied for decades, with conflicting conclusions reached. Wheeler et al. reported high levels of SpeB protease production among 73% of GAS strains cultured from pediatric patients with bacteremia (73). Holm et al. and Norrby et al. reported high levels of SpeB production among *emm1* strains from patients in Scandinavian countries with invasive GAS infections (45, 74). Norgren et al. studied 38 serotype *emm1* strains cultured from the blood of patients with serious GAS infections and also reported high levels of SpeB production (75). Talkington et al. analyzed 62 strains cultured from patients with invasive infections, including 32 with streptococcal toxic shock syndrome, and found that *emm1* strains were significantly associated with protease production (76). Moreover, protease production was significantly associated with soft tissue necrosis, including myositis and fasciitis (76). Chaussee et al. reported a lack of significant association between SpeB protease production and the severity of human infection based on analysis of strains of various *emm* serotypes cultured from 112 patients (77). Chatellier et al. analyzed 35 patients with invasive disease with different levels of severity caused by clonally related serotype *emm1* strains and failed to identify a significant relationship between the level of SpeB protease production and disease severity (78). In contrast, based on the level of SpeB protease activity made *in vitro* by serotype *emm1* strains, Kansal et al. reported an inverse relationship between disease severity and SpeB expression (23). No data bearing on host factors, such as the level of anti-SpeB antibody present in acute-phase sera from these patients, were reported (23). Similarly, SpeB-negative mutants can be selected rapidly and rise to numerical prominence in experimentally infected mice (33). However, pathogen-host interactions in mice do not necessarily translate to the common human condition (79, 80). In support of a possible host (mouse compared to human) species effect on *covRS* polymorphism acquisition, we sequenced the genomes of 2,954 *emm1* GAS strains recovered from 4 nonhuman primates (the most human-relevant animal model possible) with experimentally induced necrotizing fasciitis. None developed mutations in any gene needed for GAS to produce wild-type SpeB, including *covRS*. Taken together, our data show that SpeB-deficient organisms have not become predominate members of globally circulating GAS populations, including strains of multiple serotypes and strains recovered in either pharyngitis or invasive infections (Fig. 1 and 2). We interpret our data, and those presented by many other investigators based on divergent lines of research (9, 22, 34–41, 43–45, 48, 73–78), as confirming the important role of SpeB in pathogen-host interaction and GAS pathogenesis (Fig. 3).

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