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An N-Terminal Signal Peptide Of Vfr Protein Negatively Influences RopB-Dependent SpeB Expression and Attenuates Virulence in *Streptococcus pyogenes*

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SUMMARY

Streptococcal pyrogenic exotoxin B (SpeB) is an extracellular cysteine protease that is a critical virulence factor made by the major human pathogen group A *Streptococcus* (GAS). *speB* expression is dependent on the regulator of proteinase B (RopB) and is upregulated with increasing cell density and during infection. Because computer modeling suggested significant structural similarity between RopB and peptide-sensing regulatory proteins made by other Gram-positive bacteria, we hypothesized that *speB* expression is influenced by RopB-peptide interactions. Inactivation of the gene (*vfr*) encoding the virulence factor related (Vfr) protein resulted in increased *speB* transcript level during the exponential growth phase, whereas provision of only the amino-terminal region of Vfr comprising the secretion signal sequence *in trans* restored a wild-type *speB* expression profile. Addition of the culture supernatant from a Vfr signal peptide-expressing GAS strain restored wild-type *speB* transcript level to a *vfr*-inactivated isogenic mutant strain. A distinct peptide in the Vfr secretion signal sequence specifically bound to recombinant RopB. Finally, overexpression of the Vfr secretion signal sequence significantly decreased *speB* transcript level and attenuated GAS virulence in two mouse models of invasive infection. Taken together, these data delineate a previously unknown small peptide-mediated regulatory system that controls GAS virulence factor production.

Keywords

Virulence; gene regulation; SpeB; RopB; signal peptide

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Introduction

Precise temporal regulation of virulence factor production is critical to microbial pathogenesis (Liu *et al.*, 2011, Somerville & Proctor, 2009, Yoon *et al.*, 2009). The genomic era has brought significant advances in understanding the range of genes influenced by specific transcriptional regulators (Goodman & Lory, 2004). However, information regarding the molecular mechanisms by which stand-alone transcription factors respond to environmental stimuli to fine tune expression of virulence-factor encoding genes is limited (Declerck *et al.*, 2007, Withers *et al.*, 2001). A better understanding of how pathogenic microbes regulate virulence factor production is critical to the goal of designing novel antimicrobials that can interfere with such signaling pathways (George *et al.*, 2008, Hung *et al.*, 2005, Kreikemeyer *et al.*, 2003, Rasko *et al.*, 2008).

Group A *Streptococcus* (GAS) is a Gram-positive bacterium that has long served as a model pathogen for investigating bacterial virulence factor regulation (Kreikemeyer et al., 2003, McIver, 2009). GAS causes a broad spectrum of human infections ranging from mild pharyngitis and impetigo to life threatening necrotizing fasciitis and streptococcal toxic shock syndrome (Olsen *et al.*, 2009). Among the many virulence factors made by GAS, a secreted cysteine protease known as streptococcal pyrogenic exotoxin B (SpeB) is critical for virulence and dissemination of infection (Lukomski *et al.*, 1998, Lukomski *et al.*, 1999, Lukomski *et al.*, 1997, Svensson *et al.*, 2000). SpeB production is growth-phase dependent and increases as GAS transitions from the exponential to the stationary growth phase (Neely *et al.*, 2003). Moreover, *speB* transcript is significantly increased during infection compared to growth in a standard laboratory medium (Loughman & Caparon, 2006). Given its significance in GAS pathogenesis, SpeB is subject to multiple layers of temporal and environmental regulation (Carroll & Musser, 2011).

Transcription of *speB* is directly controlled by a global regulator known as regulator of proteinase B (RopB) (Chaussee *et al.*, 1999, Lyon et al., 1998, Carroll & Musser, 2011). RopB belongs to the Rgg-family of transcription regulators that are present in a diverse array of pathogenic low G+C Gram-positive bacteria (McIver, 2009). The *speB* promoter region has various *cis*-acting elements including two putative RopB binding sites with pseudo-palindromic sequences that are separated from each other by approximately ~120 bp (Fig. 1A) (Neely et al., 2003). RopB is required for activation of *speB* transcription, but attempts to activate *speB* expression at exponential phase of growth by ectopic provision of RopB failed to produce early onset of *speB* expression (Neely et al., 2003). Thus, it appears that additional signals are required for temporal regulation of SpeB production, but the identity and nature of such signals remain poorly understood (Neely et al., 2003, Chaussee *et al.*, 1997).

To better understand the molecular mechanism of gene regulation by RopB, we recently performed three-dimensional computer modeling of RopB (Carroll *et al.*, 2011). We discovered that RopB has significant predicted structural homology with PlcR from *Bacillus cereus* and PrgX from *Enterococcus faecalis*, the founding members of the Rap/Npr/PlcR/PrgX (RNPP)-family of quorum-sensing regulators (Fig. 1B) (Rocha-Estrada *et al.*, 2010). Members of the RNPP-family use secreted peptides as regulatory signals and alter gene

expression in response to changes in bacterial cell density (Mashburn-Warren *et al.*, 2010, Rocha-Estrada et al., 2010, Fleuchot *et al.*, 2011, Fontaine *et al.*, 2010, Declerck et al., 2007). Given its predicted structural homology with PlcR and PrgX, it is possible that RopB uses secreted peptides as regulatory signals to control *speB* expression. In this regard, it is noteworthy that 15 years ago it was reported that inactivation of the GAS genes encoding oligopeptide permeases (Opp) or dipeptide permeases (Dpp) drastically reduced *speB* expression (Podbielski *et al.*, 1996, Podbielski & Leonard, 1998) suggesting that peptide transport plays a role in *speB* regulation.

Ma et al. (2009) recently reported that inactivation of the gene (*vfr*) encoding virulence factor related (Vfr) protein increased *speB* expression and decoupled *speB* expression from growth-phase dependency (Ma *et al.*, 2009). Vfr lacks typical characteristics of a DNA binding protein and the molecular mechanism by which it alters SpeB production is not known. Vfr contains a putative amino-terminal secretion signal sequence (Ma *et al.*, 2009). After processing by peptidases, signal peptides in the extracellular milieu can be imported to the cytosol where they interact with transcriptional regulators (Kozlowicz *et al.*, 2006, Antiporta & Dunny, 2002, Lazazzera, 2001). In this study we tested the hypothesis that a peptide derived from the amino-terminal region of the Vfr secretion signal sequence directly interacts with RopB to regulate SpeB production. Our findings confirmed this hypothesis and elucidated a new mechanism of GAS virulence factor regulation.

Results

Kinetics of *vfr, ropB and speB* transcript levels in a serotype M3 GAS strain

The only previous study of the *vfr* gene reported that its transcript peaks in the exponential growth phase and gradually decreases in stationary phase (Ma et al., 2009). Given that there is significant strain-to-strain variation in gene expression profiles among GAS isolates (Dmitriev *et al.*, 2008), we first measured the transcript levels of *vfr, ropB* and *speB* in a serotype M3 strain (MGAS10870) at different growth stages (Fig. 2A). MGAS10870 was chosen for this study because it is an invasive isolate that has been fully sequenced, and it contains the most common RopB isoform (Beres *et al.*, 2010, Carroll et al., 2011). Consistent with previous observations (Carroll et al., 2011, Neely et al., 2003), in standard laboratory medium *ropB* transcript reached a maximum level in late-exponential phase, and the *speB* transcript level peaked in the stationary phase (Fig. 2B). The kinetics of the *vfr* and *ropB* transcripts paralleled one another, reaching their highest levels at late exponential phase and returning to a basal level in stationary phase (Fig. 2B). These data suggest that the presence of Vfr in the late exponential growth phase may be inhibiting RopB-mediated *speB* transcription, and this inhibition is subsequently relieved in the stationary phase of growth.

Vfr negatively influences *speB* expression in the late-exponential growth phase

To investigate the role of *vfr* in regulation of *speB* expression, we used non-polar insertional mutagenesis to construct an isogenic mutant derivative of strain MGAS10870 in which the entire *vfr* gene was replaced by a spectinomycin-resistance cassette. For the purpose of clarity, strain 10870Δ*vfr* will be referred to hereafter as Δ*vfr*. The growth kinetics of strains MGAS10870 and Δ*vfr* did not differ significantly in rich laboratory media (Fig. 2A). Next,

we compared *speB* transcript levels of the wild-type and mutant strains at different phases of growth. Very little *speB* transcript was expressed in the mid-exponential growth phase by either strain MGAS10870 or strain Δ*vfr* (Fig. 2C). However, compared to wild-type, strain Δ*vfr* had an approximately 64-fold increase in the *speB* transcript level at the late-exponential growth phase (Fig. 2C). The *speB* transcript level in mutant strain Δ*vfr* remained elevated in the stationary-growth phase, but the difference at this point was only about two-fold compared to wild-type (Fig. 2C).

To determine if these differences in transcript level resulted in variation in the amount of SpeB made by the strains, we assessed the presence of secreted SpeB in the extracellular medium by Western immunoblotting and SpeB protease activity via by an azocasein-cleavage assay. Consistent with the transcript data, immunoreactive SpeB was detected only in the growth medium of strain Δ*vfr* at the late-exponential growth phase, whereas SpeB was detected in the growth medium of both strains at the stationary phase (Fig. 2D). Next, we measured the SpeB protease activity by a chromogenic azocasein hydrolysis assay. Strain Δ*vfr* had increased SpeB protease activity in the late-exponential and stationary growth phase compared to wild-type, although the differences observed at the stationary growth phase were more pronounced compared to those observed at the late-exponential growth phase (Fig. 2E). Taken together, these data indicate that, directly or indirectly, Vfr negatively influences *speB* expression and SpeB production, with the majority of the regulatory effect occurring in the late-exponential growth phase.

Overexpression of Vfr negatively influences *speB* expression

To test the hypothesis that ectopic expression of *vfr* by a plasmid would complement the *vfr*-inactivated isogenic mutant strain, we constructed plasmid *pDC-vfr* containing the entire *vfr* gene and its promoter region. The complemented mutant strain (strain Δ*vfr*:*pDC-vfr*), strain Δ*vfr* with empty vector (strain Δ*vfr:pDC*), and the wild-type strain with empty vector (strain 10870: *pDC*) did not differ significantly in growth (Fig. 3A). To ensure that *vfr* in the *pDC-vfr* construct constituted a functional transcriptional unit, we measured the level of *vfr* transcript in the complemented strain by qRT-PCR. The pattern of the relative abundance of *vfr* transcripts at different growth stages was preserved in the complemented strain. We also found an overall increase (≥16 fold) in *vfr* transcript level in the complemented strain relative to wild-type strain MGAS10870, indicating that the complemented strain overexpressed *vfr* transcript (Fig. 3B). Importantly, the presence of the *vfr*-overexpressing plasmid in the *vfr*-inactivated strain restored a *speB* phenotype similar to that of wild-type at the late-exponential growth phase (Fig. 3C). Suppression of *speB* transcription by *pDC:vfr* was extended well into the stationary phase of growth, as the increased *speB* transcript level characteristic of stationary phase was absent in strain Δ*vfr*:*pDC-vfr* (Fig. 3C). Consistent with the qRT-PCR data, no detectable immunoreactive SpeB was made by the complemented mutant strain (Fig. 3D). Similarly, negligible SpeB protease activity was detected in culture supernatants prepared from strain Δ*vfr*:*pDC-vfr* samples with levels being comparable to those of the isogenic mutant SpeB-inactivated strain (strain 10870Δ*speB*) (Fig. 3E). Thus, the *speB* phenotype of the Vfr-inactivated strain can be reversed by *trans*-complementation with *vfr*. In addition, the negative regulatory influences of Vfr on *speB* can be extended into stationary growth phase by *vfr* overexpression.

Overexpression of only the amino-terminal secretion signal sequence of Vfr negatively influences *speB* expression

To test the hypothesis that the element in Vfr responsible for regulating SpeB resides in the amino-terminal secretion signal sequence, we cloned the *vfr* gene region that encodes only the amino-terminal 39 amino acid residues of Vfr and the *vfr* promoter region into *pDC123* to create plasmid *pDC-pep*. Consistent with our hypothesis, the complemented strain Δ*vfr:pDC-pep* had a *speB* transcript level profile essentially identical to that observed for the wild-type strain containing empty vector (Fig. 3C). The decreased *speB* transcript level observed in the late-exponential growth phase for strain Δ*vfr:pDC-pep* was comparable to that of full-length *vfr* complementation. These results indicate that the secretion signal peptide of Vfr alone is sufficient to negatively influence *speB* expression at the late-exponential growth phase (Fig. 3C). Similarly, results from western immunblotting analysis showed that SpeB production in strain Δ*vfr:pDC-pep* was comparable to wild-type in the late exponential phase but there was decreased production of the mature SpeB isoform in strain Δ*vfr:pDC-pep* in the stationary phase (Fig. 3D). The azocasein cleavage assay was too insensitive to reveal differences in SpeB protease activity in exponential-phase samples (Fig. 2E). Thus, we performed the assay only with stationary-phase samples. Compared to the *vfr*-inactivated strain containing empty vector, SpeB protease activity was significantly reduced at stationary phase in both complemented strains, with full-length complementation causing more pronounced regulation (Fig. 3E). In the aggregate, these data show that the amino-terminal secretion signal sequence of Vfr is sufficient for the negative influence of Vfr on *speB* expression in the late exponential phase of growth.

A factor in the culture supernatant of a Vfr-expressing strain restores a wild-type *speB* phenotype to strain Δ*vfr*

Multiple attempts to synthesize peptides corresponding to regions of the Vfr secretion signal sequence failed to yield synthetic peptide of reliable sequence, high purity, high yield, and solubility, likely due to marked hydrophobicity of the peptides. Thus, we could not investigate whether direct addition of a synthetic peptide containing the Vfr secretion signal sequence to the growth medium inhibited *speB* expression. Rather, we performed culture supernatant-swapping experiments designed to determine if the Vfr secretion signal peptide is present in the extracellular environment and then internalized. Strain Δ*vfr* was grown to mid-exponential phase (A_{600} ~0.6), cells were collected by centrifugation, and the cell pellet was suspended in the filtered growth media prepared from late-exponential phase cultures (A_{600} ~0.8) from one of the following strains: Δ*vfr:pDC, strain* Δ*vfr:pDC-vfr, or* Δ*vfr:pDC-pep.* No immunoreactive SpeB was present in the pre-swap samples (data not shown). Samples were collected 1 h post-swapping (A_{600} ~1.0), and *speB* transcript level and secreted SpeB were measured by qRT-PCR and Western immunoblotting, respectively. Consistent with the hypothesis that the secretion signal sequence of Vfr is present in the extracellular milieu and subsequently imported into the cytoplasm to mediate *speB* regulation, significantly less *speB* transcript level was observed in strain Δ*vfr* grown in the medium derived from strain Δ*vfr:pDC-vfr* or strain Δ*vfr:pDC-pep* compared to the same strain exposed to the medium derived from strain Δ*vfr-pDC* (Fig. 4A and 4B). Similarly, culture supernatant derived from strain Δ*vfr:pDC-vfr or strain* Δ*vfr:pDC-pep*, but not strain

Δ*vfr:pDC*, drastically reduced the production of immunoreactive SpeB (Fig. 4B). Thus, *speB* expression is negatively influenced by Vfr or a product derived from it that is present in the culture supernatant.

To determine the chemical nature and the likely molecular mass of the regulatory factor, we performed a swap assay with culture supernatants of Δ*vfr:pDC-vfr* that is sieved through YM-3 (~3 kDa molecular weight cut-off filter) membrane filter and/or treated with proteinase K. In accordance with the hypothesis that the secreted regulatory factor has low molecular mass, strain Δ*vfr* grown in the low molecular weight component (≤ 3 kDa) of the culture supernatant obtained from Δ*vfr:pDC-vfr* strain had negligible levels of secreted SpeB compared to that of strain Δ*vfr* grown in the low molecular weight component of supernatant derived from strain Δ*vfr:pDC* (Fig. 4C). Consistent with the hypothesis that the regulatory factor present in the culture supernatant of Δ*vfr:pDC-vfr* is proteinaceous, strain Δ*vfr* grown in the proteinase K treated culture supernatant of Δ*vfr:pDC-vfr* failed to repress SpeB production (Fig. 4C). Together, these data indicate that the secreted factor present in the culture supernatant of Δ*vfr:pDC-vfr* that negatively regulates *speB* expression is a low molecular weight peptide.

A distinct region of the amino-terminal secretion signal sequence of Vfr interacts with RopB

Inasmuch as the predicted structural homologs of RopB interact with secreted peptides to mediate gene regulation (Shi *et al.*, 2005, Declerck et al., 2007), we next investigated whether a distinct region of the amino-terminal signal sequence of Vfr directly interacts with RopB using a synthetic peptide array. Overlapping 17-amino acid synthetic peptides that span the entire predicted signal sequence of Vfr (amino acid residues 1-49) were generated by SPOT synthesis on a cellulose membrane. The resulting synthetic peptide array encompassed all three components commonly found in secretion signal sequences, namely a basic n-region, a hydrophobic h-region, and a c-region with the protease cleavage site (Fig. 5A). Interaction between the synthetic peptides and purified recombinant hexa-histidine-tagged RopB (Fig. 5B) was assessed. The data show that RopB interacts with a distinct region of the amino-terminal secretion signal sequences of Vfr (Fig. 5C). The amino acids required for RopB binding are located in the h-region and c-region fragment of the Vfr signal sequence (Fig. 5C). Omission of amino acid residues R39 at the carboxy-terminus and L32 at the amino-terminus markedly decreased RopB binding, indicating the likely boundaries of the RopB binding site in the Vfr signal sequence (Fig. 5C). Thus, we conclude that a region of the amino-terminal secretion signal sequence of Vfr interacts directly with RopB suggesting a mechanism by which Vfr alters *speB* expression.

Overexpression of the *vfr* secretion signal sequence significantly decreases GAS virulence in mouse models of invasive infection

To test the hypothesis that the *vfr* gene and its influence on *speB* expression contribute to GAS virulence, we compared strains MGAS10870, Δ*speB*, Δ*vfr*, Δ*vfr:pDC-vfr*, and Δ*vfr:pDC-pep* in two different mouse models of invasive infection. First, mice were injected intraperitoneally with 1×10^7 colony-forming units (CFU) of each strain, and the ability to cause near mortality was monitored for seven days. The strains could be divided into two

groups based on virulence phenotype. Wild-type strain MGAS10870 and mutant strain Δ*vfr* caused significantly higher mortality compared to strains Δ*vfr:pDC-vfr*, Δ*vfr:pDC-pep*, or Δ*speB* ($P < 0.01$ when comparing any two strains in different groups) (Fig. 6A). There was no significant difference in mortality among strains within either of the two groups.

GAS cause a wide range of human infections with different virulence factors having varying impact depending on the site of infection (Engleberg *et al.*, 2004, Olsen et al., 2009). Therefore, we also tested the role of *vfr* in GAS virulence using an intramuscular infection model that mimics human necrotizing fasciitis. Consistent with the results obtained from the intraperitoneal injection experiments, strains MGAS10870 and Δ*vfr* were significantly more virulent than strains Δ*vfr:pDC-pep*, Δ*vfr:pDC-vfr*, and Δ*speB* ($P < 0.01$ when comparing any two strains in different groups) (Fig. 6B). To better understand the role of *vfr* in GAS pathogenesis at the cellular level, we performed histopathologic analysis of intramuscular tissue lesions. The strains from the two groups caused contrasting pathologic characteristics. Lesions from the mice infected with strains MGAS10870 and Δ*vfr* showed severe host-tissue damage and disseminated infection (Fig. 6C). In comparison, lesions from mice infected with strains Δ*vfr:pDC-pep*, Δ*vfr:pDC-vfr*, and Δ*speB* were small, circumscribed and bordered by healthy muscle tissue (Fig. 6C).

To gain insight into the molecular mechanism by which overexpression of the Vfr secretion signal sequence reduces GAS virulence *in vivo*, we performed qRT-PCR analysis for *speB* and *vfr* transcript levels in infected mouse tissue. Similar to our observations for the stationary phase of growth in standard laboratory medium (Fig. 2C), there was no significant difference in *in vivo speB* transcript level between wild-type strain MGAS10870 and mutant strain Δ*vfr* (Fig. 6D). In contrast, there was a significant decrease in *speB* transcript level in strains Δ*vfr:pDC-vfr* and Δ*vfr:pDC-pep* (Fig. 6D). As expected, no detectable *vfr* transcript was made by strain Δ*vfr*, whereas *vfr* transcripts were present at a relatively high level in both complemented strains, Δ*vfr:pDC-vfr* and Δ*vfr:pDC-pep* (Fig. 6E). Interestingly, *vfr* transcript level was nearly undetectable in strain MGAS10870 suggesting strong repression of *vfr* expression during infection (Fig. 6E). Together, these data indicate that inactivation of Vfr does not significantly affect GAS virulence but overexpression of the Vfr secretion signal sequence significantly reduces *speB* transcript level in the host and attenuates GAS virulence.

Discussion

Many bacterial pathogens sense changes in population density in their environment via secreted molecules and respond by altering expression of genes involved in diverse cellular processes such as competence, conjugation, biofilm formation, and pathogen-host interaction (Bassler & Losick, 2006). Virulence gene expression at high cell density can result in host tissue damage and promote microbial dissemination and survival (Bassler, 1999, Van Delden & Comte, 2001, De Kievit & Iglewski, 2000). Conversely, pathogens also have mechanisms to downregulate virulence gene expression at low cell density to avoid premature elicitation of host defense responses which could promote bacterial clearance from the site of infection (Kozlowicz *et al.*, 2006). The data presented herein provide new insights into virulence gene regulation in response to changes in the GAS growth phase. We

have shown that at low cell density and in a growth-phase dependent manner, the amino-terminal secretion signal sequence of Vfr negatively influences expression of a key virulence factor, SpeB, by interfering with RopB-dependent *speB* transcription.

A previous study reported that genetic inactivation of *vfr* relieves growth-phase-dependent repression of *speB* expression (Ma et al., 2009). It was proposed that the Vfr influence occurs through protein-protein interactions with molecules in other regulatory networks (Ma et al., 2009). Our data show that the SpeB-related phenotype in Vfr inactivated strain can be reversed with only the region of *vfr* encoding the secretion signal sequence. This finding indicates that the *speB* regulatory function of *vfr* resides in the amino-terminal 40 amino acids of Vfr. Known regulatory peptides in Gram-positive bacteria are generally derived from either a small peptide-encoding open reading frame or from the amino-terminal secretion signal sequence of lipoproteins (Lazazzera, 2001, Antiporta & Dunny, 2002). In this regard, we note that the Vfr propeptide region has many of the characteristics of Gram-positive pheromones including a basic amino acid-rich amino-terminus region (n-region) and a hydrophobic central region (h-region). However, the Vfr secretion signal sequence lacks a carboxy-terminal cysteine residue commonly required for signal peptidase II cleavage and secretion (Paetzel *et al.*, 2002, Von Heijne, 1990) (Fig. 5A). Interestingly, recently identified pheromone propeptides from *S. mutans* and *S. thermophilus* also lack the characteristic cysteine residue at the cleavage site and are believed to be processed and secreted by unknown secretion systems (Fontaine et al., 2010, Mashburn-Warren et al., 2010). Additionally, Chang et.al recently reported that short-hydrophobic peptides (SHP2 and SHP3) interact with RopB-like regulators (designated Rgg2 and Rgg3 in that study) in GAS and mediate gene regulation (Chang *et al.*, 2011). The Vfr-RopB interaction seems fundamentally distinct from those of the SHP-Rgg as *shp2* and *shp3* are encoded immediately upstream of the respective *rgg* gene and are not part of a larger open reading frame as is the case with the Vfr N-terminal signal sequence. Thus, in concert with recently published data, our findings add to the rapidly expanding appreciation of the role of the diverse role of small peptide molecules in GAS pathophysiology.

Similar to many other bacterial transcription regulators, RopB may function as a dimer, and two RopB binding sites with inverted repeats have been identified in the *speB* promoter region that are separated from each other by ~130 bp (Fig. 1B) (Neely et al., 2003). For both PrgX and PlcR (structural homologs of RopB) peptide-binding triggers structural changes in their respective effector molecule that leads to altered DNA-binding and/or oligomerization, thereby altering gene expression (Kozlowicz et al., 2006, Shi et al., 2005, Declerck et al., 2007). PlcR-promoter interactions and the consequent transcription activation by PlcR occur only in the presence of the activating peptide PapR (Slamti & Lereclus, 2002). On the other hand, binding of either inhibitory peptide, iCF10 or activating peptide, cCF10 to PrgX controls the regulatory properties of PrgX. Interaction between PrgX and iCF10 promote PrgX tetramerization, resulting in the occupation of both PrgX binding sites by DNA looping and repression (Kozlowicz et al., 2006). Conversely, interactions between cCF10 and PrgX disrupt the tetramerization interface and the dimeric PrgX activates gene expression (Shi et al., 2005). Thus, the Vfr regulatory peptide may influence *speB*

expression by modulating oligomerization of RopB, thereby interfering with or otherwise altering DNA-binding at one or both of the *speB* promoter binding sites.

The negative regulation of *speB* expression by Vfr is more pronounced during the exponential phase of growth, with repression being relieved in the stationary growth phase. Our data indicate that removal of Vfr-mediated negative regulation of *speB* expression by downregulation of *vfr* gene expression at stationary phase contributes to transcriptional activation of *speB* (Fig. 2B). Similarly, repression of *vfr* transcript level during infection may help to explain the high levels of *speB* transcript that are typically observed following GAS infection (Fig. 6D, 6E). However, inactivation of *vfr* alone cannot fully account for the rapid increase in *speB* expression observed in MGAS10870 at stationary-growth phase as the Vfrinactivated strain had the same *speB* transcript level as strain MGAS10870 at the mid-exponential growth phase (Fig. 2C). Moreover, the *speB* transcript level in strain Δ*vfr* at late-exponential phase remained significantly less than that observed for strain MGAS10870 at the stationary phase of growth. Thus it is likely that an activation element, possibly a secreted peptide that is produced at high cell density is responsible for the activation of *speB* expression observed at stationary phase. Similar dynamics between inhibiting and activating peptides were observed in the regulation of PrgX function (Shi et al., 2005, Kozlowicz et al., 2006). The finding of such an activation peptide would explain the long-standing observation that elimination of transporter systems involved in the uptake of small peptides reduces SpeB production and might help explain how GAS is able to increase SpeB production during infection (Podbielski *et al.*, 1996, Podbielski & Leonard, 1998).

In conclusion, we show that the amino-terminal secretion signal sequence of Vfr is an integral part of the system used by GAS to regulate expression of the gene encoding SpeB, a critical virulence factor. These data describe the existence of a pheromone-like regulatory circuit that controls virulence gene expression in a growth-phase depending manner. Complete elucidation of peptide-controlled regulatory networks such as this could serve as the platform for the development of novel intervention strategies.

Experimental Procedures

Bacterial strains, plasmids and growth conditions

Bacterial strains and plasmids used in this study are listed in Table 1. Strain MGAS10870 is a previously-described invasive serotype M3 isolate whose genome has been fully sequenced (Beres et al., 2010). MGAS10870 is representative of serotype M3 strains that cause invasive infections and has a wild-type sequence for all major regulatory genes including RopB (Beres et al., 2010). GAS was grown routinely on Trypticase Soy agar containing 5% sheep blood (BSA; Becton Dickinson) or in Todd-Hewitt broth containing 0.2% (w/v) yeast extract (THY; DIFCO). When required, spectinomycin or chloramphenicol was added to a final concentration of 150 μg/ml and 5 μg/ml, respectively. All GAS growth experiments were done in triplicate on three separate occasions for a total of nine replicates. Overnight cultures were inoculated into fresh medium to achieve an initial OD_{600} of 0.03. Growth was monitored by measuring A_{600}. The *Escherichia coli* strain used for protein overexpression was grown in Luria-Bertani broth (LB broth; Fisher Scientific) and when appropriate ampicillin was added to a final concentration of 40 μg/ml.

Creation of *vfr*- and *speB*-inactivated mutant strains

Insertion inactivation of the *speB* and *vfr* genes in wild-type strain MGAS10870 was performed by methods described previously (Kuwayama *et al.*, 2002, Lukomski *et al.*, 2000). Briefly, a PCR fragment containing a spectinomycin-resistance (*spc*) cassette with the fragment of gene to be deleted on either side was generated in a three-step PCR process. Subsequently, the plasmid with the *spc* gene-disruption cassette was introduced into the parent strain by electroporation and the gene was disrupted through homologous recombination. The isogenic mutant strains were selected by growth on spectinomycin-containing medium. Inactivation of the gene was confirmed by DNA sequencing. Primers used for the construction of strain 10870Δ*speB* and Δ*vfr* are listed in supplemental Table S1.

Construction of Vfr full-length and Vfr-peptide complementation plasmids

To complement isogenic mutant strain Δ*vfr*, the coding sequence of the full-length *vfr* gene or the region encoding the amino-terminus secretion signal sequence (amino acid residues 1-39) were cloned into the *E. coli*-GAS shuttle vector *pDC123* (Chaffin & Rubens, 1998): Using the primers listed in Table S1, the respective fragments were amplified by PCR from GAS genomic DNA, digested with *Bgl*II and *Nde*I, and ligated into digested vector pDC123. The inserts were verified by DNA sequencing and electroporated into the appropriate mutant derivatives of strain MGAS10870.

Measurement of transcript levels by quantitative RT-PCR

GAS strains were grown to the indicated OD_{600} and incubated with two volumes of RNAprotect (Qiagen) for 10 min at room temperature. Bacteria were harvested by centrifugation and the cell pellets were snap frozen with liquid nitrogen. RNA isolation and purification were performed using an RNeasy kit (Qiagen). Purified RNA was analyzed for quality and concentration with an Agilent 2100 Bioanalyzer. cDNA was synthesized from the purified RNA using Superscript III (Invitrogen) and Taqman quantitative RT-PCR was performed with an ABI 7500 Fast System (Applied Biosystems). Comparison of transcript levels was done using ΔC_T method of analysis using *tufA* as the endogenous control gene (Virtaneva *et al.*, 2005). The Taqman primers and probes used are listed in Table S1.

Western immunoblot analysis of SpeB in the culture supernatant

Cells were grown to the indicated A_{600} and harvested by centrifugation. The culture supernatant was filtered and the filtrate was concentrated two-fold by speed-vac drying. Equal volumes of the samples were resolved on a 15% SDS-PAGE gel, transferred to a nitrocellulose membrane, and probed with polyclonal anti-SpeB rabbit antibodies (Shelburne *et al.*, 2010). SpeB was detected by secondary antibody conjugated with horseradish peroxidase and visualized by chemiluminescence using SuperSignal West Pico Rabbit IgG detection kit (Thermo Scientific).

Casein hydrolysis assay for SpeB protease activity

Cysteine protease activity in the culture supernatant was analyzed by methods described previously (Collin & Olsén, 2000). Briefly, GAS strains were grown to indicated OD_{600} and harvested by centrifugation. The culture supernatant was filtered with a 0.22 μM membrane

filter and the filtrate was used for the assay. Culture supernatant (200 μl) was mixed with an equal volume of activation buffer (0.1 M acetate buffer pH 5.0, 1 mM EDTA, and 20 mM DTT) and incubated at 40° C for 30 min. The activated sample was incubated with 400 μl of 2% azocasein (Sigma) in activation buffer for 60 min at 40° C. The reaction was stopped by adding trichloroacetic acid (TCA) to a final concentration of 10% v/v. After 5 min incubation at room temperature, the samples were vortexed and centrifuged at 15,000 rpm for 5 min. Casein hydrolysis was assessed by measuring the absorbance at 360 nm. A control sample in which TCA was added to the activated reaction before the addition of azocasein was used as blank.

Culture supernatant swapping experiments

Overnight cultures of strains Δ*vfr*, Δ*vfr:pDC*, Δ*vfr:pDC-vfr*, and Δ*vfr:pDC-pep* were inoculated into fresh THY media. The complemented strains were grown to A_{600} ~0.8, cells were pelleted by centrifugation, and culture supernatants were prepared by filtering through a 0.22 μM membrane filter. The cell pellets of strain Δ*vfr* grown to mid-exponential phase (A_{600} ~0.6) were suspended in the culture supernatants prepared from *one of the three strains tested,* Δ*vfr:pDC* or Δ*vfr:pDC-vfr or* Δ*vfr:pDC-pep* and incubated at 37°C for 1 h (A_{600} ~1.0). RNA analysis and Western immunoblotting were then performed as described above.

To characterize the regulatory factor present in the culture supernatant of Δ*vfr:pD-Cvfr*, the complemented strain was grown at 37°C to late exponential phase (A_{600} ~0.8) and the culture supernatant was collected by centrifugation and prepared as described below. To obtain the low molecular weight component of the culture supernatant, the samples were filtered using YM-3 (3 k Da cut-off filter) membrane and the flow through was used for the culture supernatant swap experiments. Proteinase K treated culture supernatants were prepared by incubating the filtered growth medium with proteinase K (0.15 mg/ml) for 1 hour at 37°C and the enzyme was subsequently removed by filtering through YM-10 (10 k Da cut-off filter) membrane. The filtered samples were used for the swapping. Swapping experiments were performed as described above.

Protein overexpression and purification

The *ropB* gene of strain MGAS10870 was cloned into plasmid pET-15b and protein was overexpressed in *E. coli* strain BL21* (DE3). Cells were grown at 37°C till the A_{600} reaches 0.5 and induced with 0.5 mM IPTG at 13°C overnight. Cell pellets were suspended in 50 ml of buffer A (20 mM Tris HCl pH 8.5, 100 mM NaCl, 10 % glycerol, and 1 mM Tris 2-carboxyethyl phosphine hydrochloride (TCEP)) supplemented with one protease inhibitor cocktail pellet and DNaseI to a final concentration of 5 μg/ml. Cells were lysed by a microfluidizer M-110L device (Microfluidics) and cell debris was removed by centrifugation at 15,000 rpm for 30 min. RopB was purified by affinity chromatography using a Ni-NTA agarose column followed by size exclusion chromatography with Superdex 200G column. The protein was purified to >95 % homogeneity by Ni-affinity chromatography (Fig. 5B).

SPOT-peptide array

Cellulose-bound peptides were prepared by automated SPOT synthesis (MultiPep RS, Intavis, Bergisch Gladbach, Germany) as described previously (Frank & Overwin, 1996, Kramer *et al.*, 1999). The SPOT membranes were rehydrated in Tris-buffered saline containing 0.05% Tween-20 (TBS-T) and blocked overnight at 4°C with 4% non-fat dry milk in TBS-T. After two washes with TBS-T, the membrane was incubated with purified hexa-histidine-tagged purified RopB at a final concentration of 2 μg/ml for 1 h at room temperature. RopB-peptide binding was assessed by chemiluminescence with anti-hexa-histidine tag antibodies conjugated to horseradish peroxidase (Clontech). Binding of the probing antibodies to the peptides was eliminated by control reactions with anti-hexahistidine tag antibody alone (data not shown).

Mouse infection studies

Virulence of the isogenic mutant GAS strains was assessed using two mouse models. For intraperitoneal infection, fifteen female 3-4 week-old CD1 mice (Harlan Laboratories) were used for each GAS strain. Animals were inoculated intraperitoneally with 1×10^7 CFUs and survival was monitored daily. Data were graphically displayed as a Kaplan-Meier survival curve and analyzed using the log-rank test. For intramuscular infection, 10 female 3-4 week-old CD1 mice (Harlan Laboratories) were inoculated in the right hindlimb with 1×10^7 CFU of each strain and monitored for near mortality. Results were graphically displayed as a Kaplan-Meier survival curve and analyzed using the log-rank test. For histopathology, infected hindlimbs were examined at 24 and 48 h post-inoculation. Tissues from excised lesions were fixed in 10% phosphate-buffered formalin, decalcified, serially sectioned, and embedded in paraffin using automated standard instruments. Hematoxylin and eosin and Gram's stained sections were examined in a blinded fashion with a BX5 microscope and photographed using a DP70 camera (Olympus). Micrographs of tissue taken from the inoculation site that showed pathology characteristic of each strain were selected for publication. All animal protocols were approved by the Institutional Animal Care and Use Committee at The Methodist Hospital Research Institute.

Transcript analysis from infected tissue

To assay *in vivo* transcript levels, skin lesions from three mice per infecting strain were collected 24 h post-infection and the tissue samples were incubated with RNAlater (Qiagen). Samples were snap frozen with liquid nitrogen and stored at −80°C until use. RNA was isolated and purified using an RNeasy fibrous tissue mini kit (Qiagen). The quality and concentration of RNA were assessed with an Agilent 2100 Bioanalyzer. cDNAs were prepared using Superscript III (Invitrogen) and transcript levels were measured by Taqman qRT-PCR. Data were analyzed using the ΔC_T method.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

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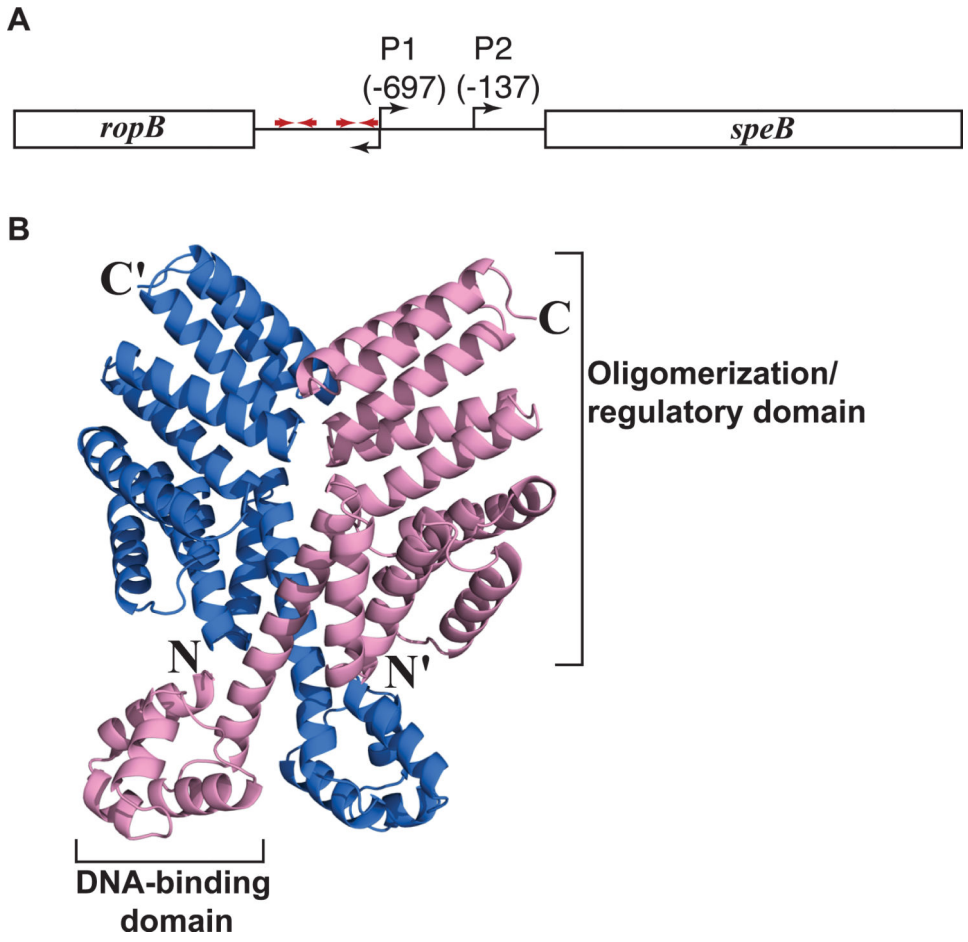

Figure 1. Organization of the *speB* gene region and RopB model

(A) Organization of the *speB* gene region in strain MGAS10870. The divergently transcribed *speB* and *ropB* genes are shown as rectangle boxes and labeled. The bent arrows above the line indicate two transcription start sites of *speB* and bent arrow below denote the transcription start site of *ropB*. Red arrows indicate putative palindromic sites that have the RopB binding site. Data regarding transcription start sites and RopB binding sites are derived from (Neely et al., 2003). (B) RopB structure predicted by I-TASSER protein modeling server (http://zhanglab.ccmb.med.umich.edu/ITASSER). Ribbon representation of the predicted RopB homodimer is shown and the individual subunits of RopB dimer are colored in blue and pink. The amino- and carboxy-terminus of one subunit are indicated as N and C, respectively, and those of the second subunit are indicated with a prime ('). The putative DNA-binding domain in the amino-terminus and the oligomerization/regulatory domain in the carboxy-terminus of one subunit are labeled. The model shown is modified from Figure 2 from (Carroll et al., 2011).

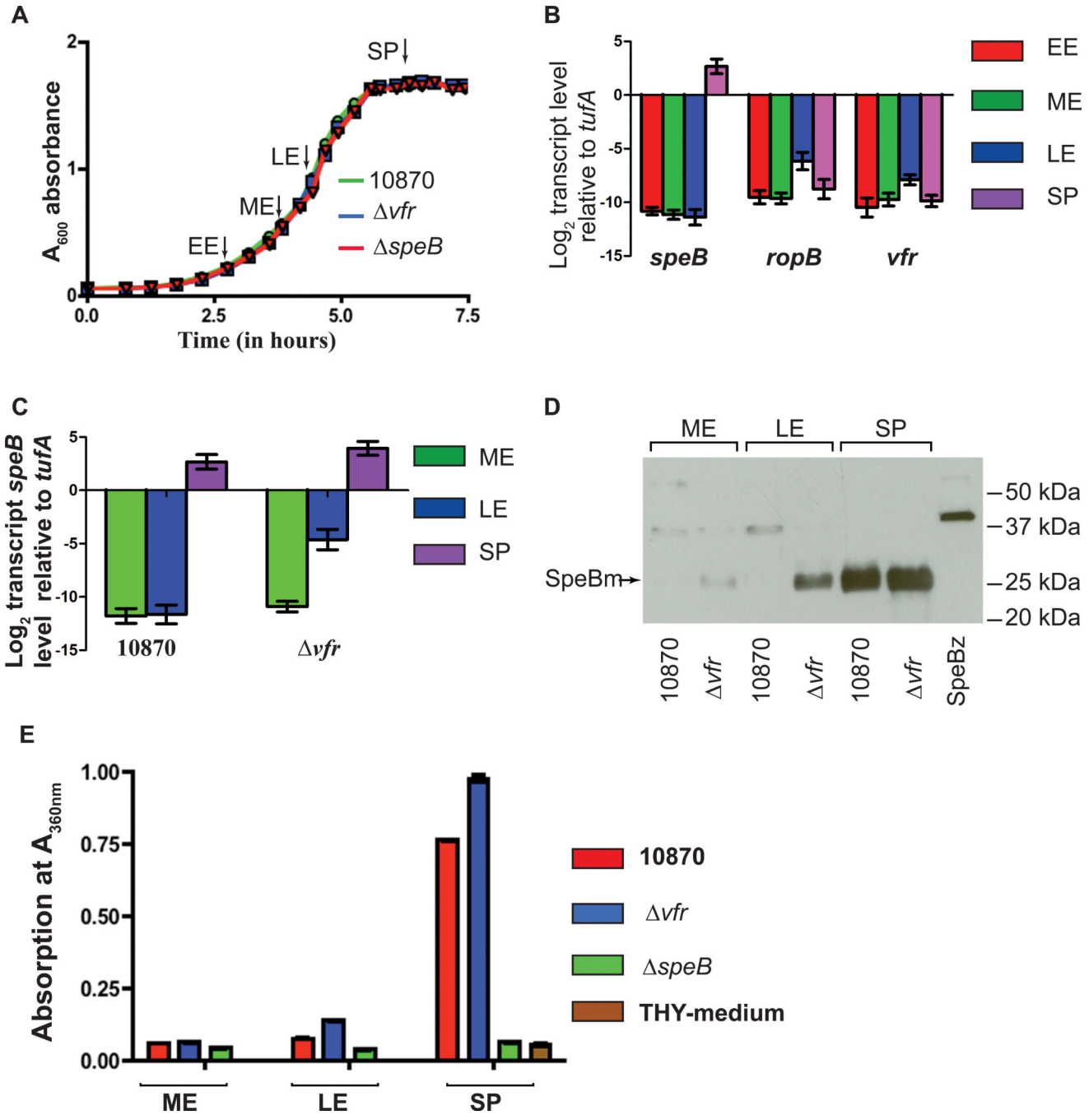

Figure 2. *vfr* inactivation alters *speB* expression profile

(A) Growth curve of indicated strains in THY broth. Samples were collected at early-exponential (EE, *(A_{600}* ~ 0.3), mid-exponential (ME, A_{600} ~ 0.6), late-exponential (LE, A_{600} ~ 1.0) and stationary phase (SP, A_{600} ~ 1.7) for transcript and protein analysis. Arrows indicated the sampling time points. (B) Transcript levels of *speB*, *ropB* and *vfr* in strain MGAS10870 measured by Taqman qRT-PCR. (C) *speB* transcript level in strains MGAS10870 and isogenic mutant strain Δ*vfr*. For (B) and (C) duplicate biological replicates were grown on two separate occasions and analyzed in duplicate. Data graphed are mean ±

standard deviation. (D) Representative western immunoblot analysis of SpeB in filtered growth media from strain MGAS10870 and isogenic mutant strain Δ*vfr*. Each experiment was performed on three different occasions with essentially identical results. Growth media collected at indicated sampling points from strains MGAS10870 and Δ*vfr* were analyzed using anti-SpeB polyclonal rabbit antibody and chemiluminescence. The bands corresponding to the mature form of SpeB are labeled (SpeBm) and indicated by an arrow. (E) Assay of SpeB protease activity by azocasein hydrolysis assay. Filtered growth media collected at indicated growth phases from strains MGAS10870 and Δ*vfr* were assayed for secreted SpeB protease activity. Azocasein hydrolysis by SpeB was determined by measuring the absorption at A_{360} nm.

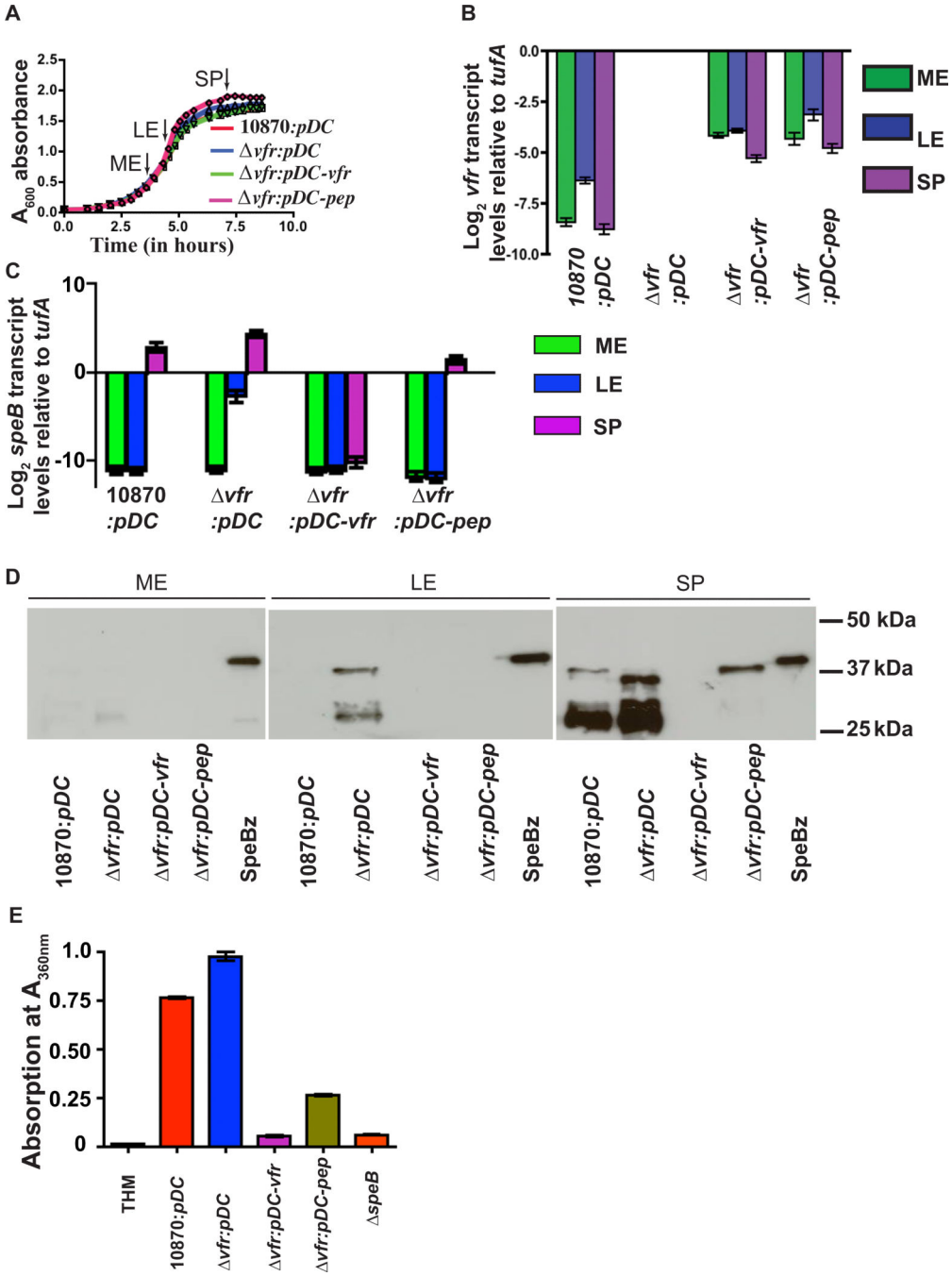

Figure 3. The secretion signal sequence of Vfr restores a wild-type speB expression profile to strain Δ*vfr*

(A) Growth pattern of indicated strains in THY broth. Samples were collected at mid-exponential (ME A_{600} ~ 0.6), late-exponential (LE A_{600} ~ 1.0) and stationary phase (SP A_{600} ~ 1.7) for transcript and protein analysis at time points designated by arrows. (B) *vfr* transcript levels as measured by Taqman qRT-PCR at indicated growth phase. (C) *speB* transcript levels in complemented strains at indicated growth phases. For (B) and (C), biological replicates were analyzed in duplicate on two separate occasions with data graphed being mean ± standard deviation. (D) Western immunoblot analysis of SpeB in filtered

growth media prepared from indicated strains at indicated growth phases. (E) Assay of SpeB protease activity in the culture supernatant by azocasein hydrolysis. Azocasein hydrolysis by SpeB was assessed by absorption at A_{360} nm. Samples were analyzed in triplicates on two separate occasions with data graphed are mean ± standard deviation.

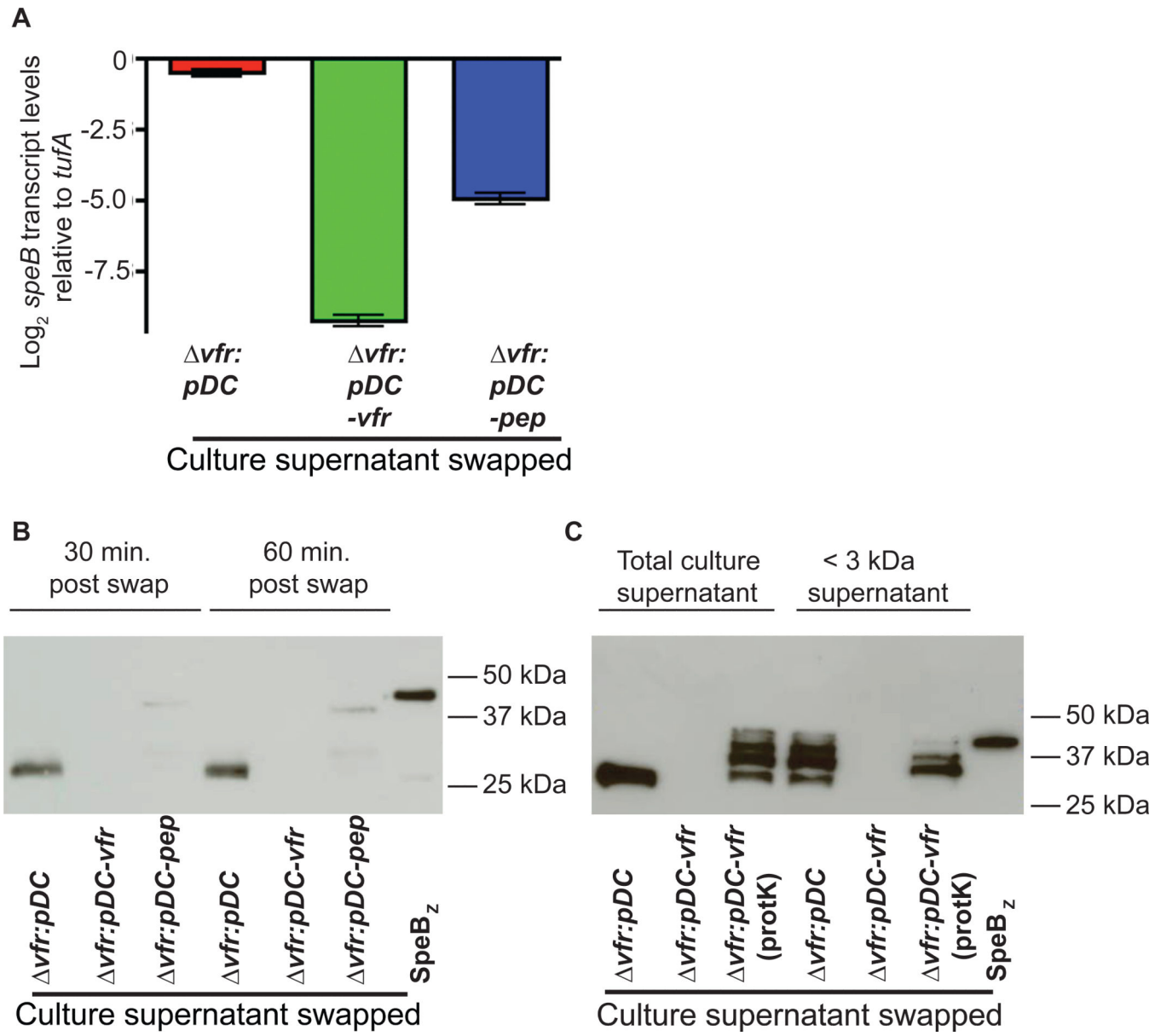

Figure 4. Vfr represses SpeB production by a product present in the culture supernatant

(A) *speB* transcript level analysis of mutant strain Δ*vfr* grown in the culture supernatant the indicated strains for 1 hr as described in Materials and Methods. Two biological replicates were performed on two separate days and analyzed in duplicate with data graphed being mean ± standard deviation. *P* value is derived from a two-sample *t*-test. (B) Western immunoblot analysis of SpeB in the growth medium of mutant strain Δ*vfr* grown for 30 minutes and 60 minutes in the culture supernatant derived from indicated strains as described in (A). *(C)* Western immunoblot analysis of SpeB in the growth medium of mutant strain Δ*vfr* grown for 60 minutes in either the total culture supernatant or culture supernatant filtered with YM-3 (< 3 kDa) membrane derived from indicated strains. Proteinase K treated culture supernatants (Prot K) used for swapping are indicated.

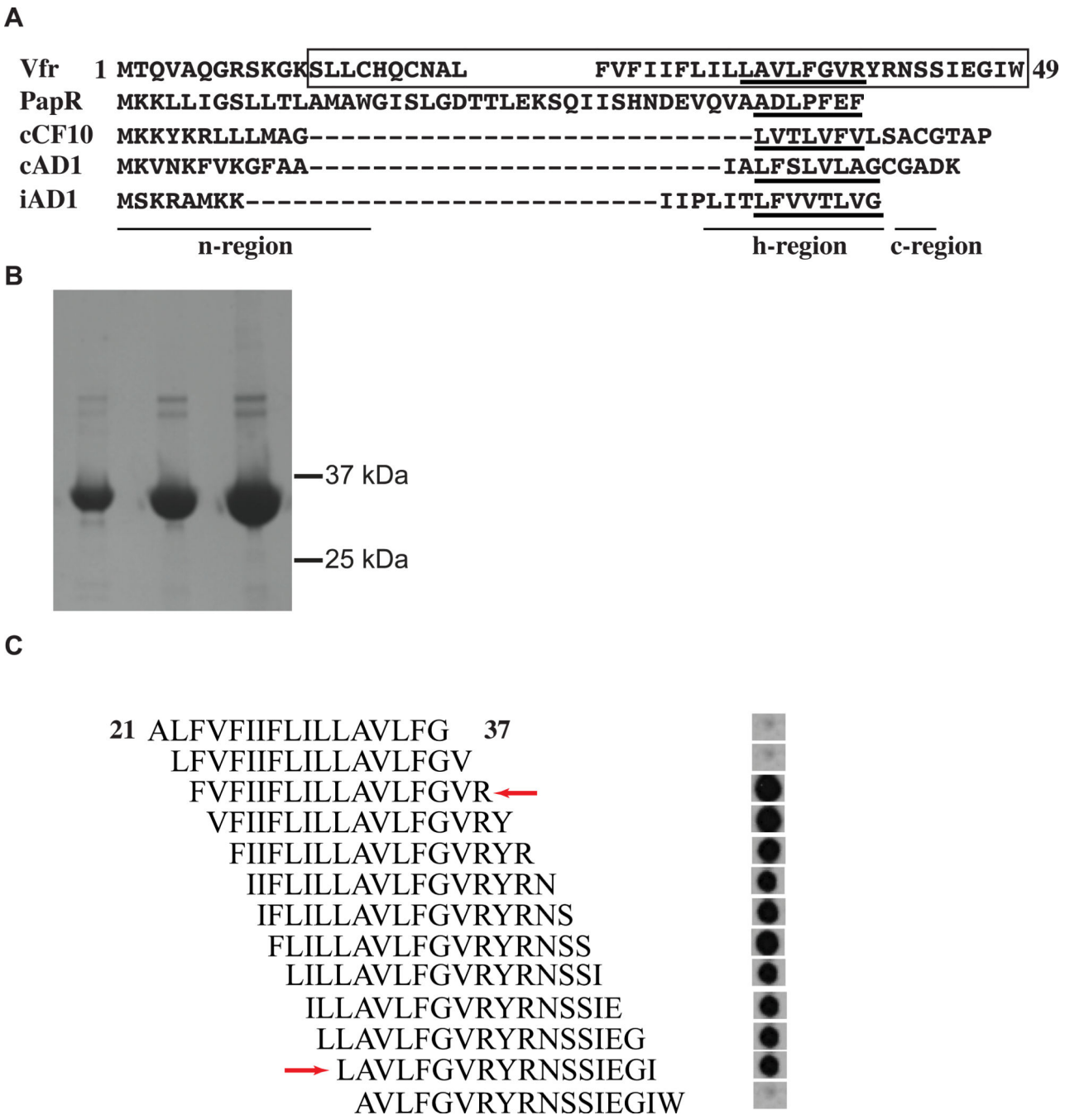

Figure 5. Recombinant RopB binds the Vfr amino-terminus signal sequence

(A) Primary sequence alignment of Vfr signal sequence with characterized Gram-positive bacterial pheromones, PapR from *Bacillus cereus*; and cCF10, cAD1, and iAD1 from *Enterococcus faecalis*. Regions corresponding to the three major components of signaling peptides, n-region, h-region, and c-region, are underlined and labeled. Amino acid sequences corresponding to mature peptides of each pheromone and the putative Vfr mature peptide were underlined. Amino acid sequences of Vfr included in the peptide array are boxed. (B) SDS-polyacrylamide gel with increasing concentrations of purified recombinant

hexa-histidine tagged RopB. The corresponding positions of molecular weight markers (in kilodaltons) are indicated. (C) Peptide array of overlapping 17-mer peptides that spans the entire putative Vfr signal sequence was generated using SPOT synthesis on a cellulose membrane. Membrane was incubated with purified recombinant hexa-histidine tagged RopB, and bound protein was detected with anti-hexahistidine tag antibodies and chemiluminescence. The red arrows indicate the likely amino-terminal and carboxy-terminal boundaries of the RopB binding site in the Vfr secretion signal sequence, located at L32 and R39 amino acid residues, respectively. The numbering of amino acid residues indicates their position within the Vfr secretion signal sequence.

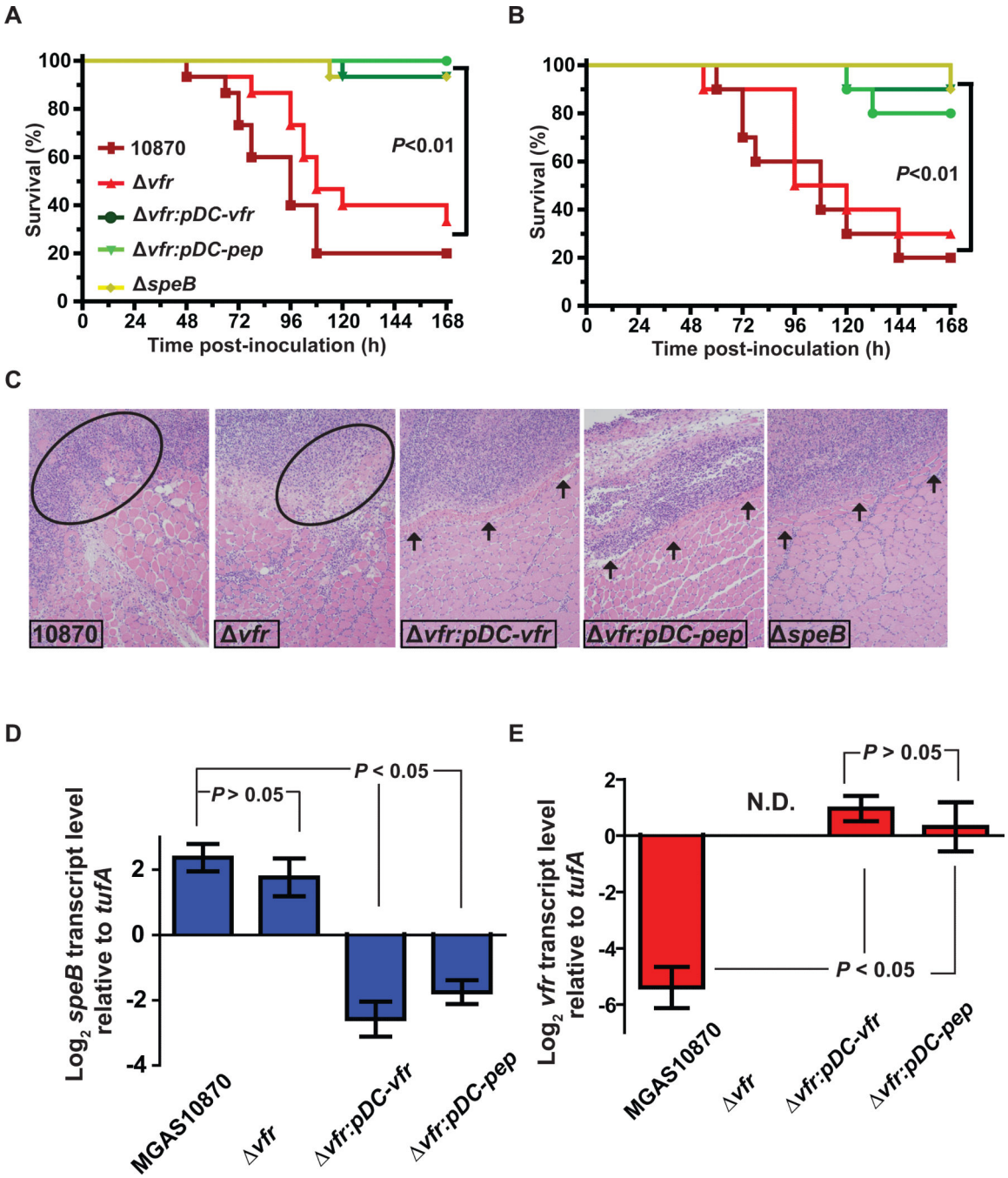

Figure 6. Overexpression of the *vfr* signal sequence decreases GAS virulence in mouse models of infection

(A) Fifteen CD-1 mice were inoculated with each indicated strain intraperitoneally and the near mortality was monitored. Kaplan-Meier survival curve with *P* values derived by log rank test. (B) Ten CD-1 mice were injected intramuscularly with 1×10^7 CFU of each strain. Kaplan-Meier survival curve with *P* values derived by log rank test. (C) Histopathologic analysis of muscular lesions from mice infected with each indicated strain. Areas of host tissue damage are circled, whereas confined, less destructive lesions are indicated by arrows. (D) and (E) TaqMan qRT-PCR analysis of *speB* and *vfr* gene transcript levels from mouse

tissue samples infected with indicated strains. Samples were collected from the lesions of three mice per infecting strain and analyzed in duplicate. Resulting data were graphed as mean ± standard deviation with *P* values derived from two-sample *t*-test.

Table 1

Bacterial strains and plasmids used in this study.

Strain or plasmid	Description	Reference
Strains		
MGAS10870	Invasive isolate, serotype M3	(Beres et al., 2010)
MGAS10870:*pDC*	MGAS10870 with empty vector, Cm^+	This study
Δ *vfr*	MGAS10870Δ*vfr*::*aad9*	This study
Δ*vfr*:*pDC*	MGAS10870Δ*vfr*::*aad9*, empty vector, Cm^+	This study
Δ*vfr-pDC*:*vfr*	MGAS10870Δ*vfr*::*aad9*, *vfr*$^+$, Cm^+	This study
Δ*vfr-pDC*:*pep*	MGAS10870Δ*vfr*::*aad9*, Vfr peptide$^+$, Cm^+	This study
MGAS10870Δ*speB*	MGAS *10870*Δ*speB*::*aad9*	This study
Plasmids		
pSL60-1	Vector containing *aad9* gene encoding spectinomycin resistance	(Lukomski et al., 2000)
pDC123	Low-copy number plasmid capable of replication in GAS and *Escherichia coli*, Cm^+	(Li *et al.*, 1997)
pDC:*vjr*	pDC123 with entire *vfr* gene plus promoter, Cm^+	This study
pDC:*pep*	pDC123 with Vfr N-terminal secretion signal plus promoter, Cm^+	This study