**vfr, a Novel Locus Affecting Cysteine Protease Production in *Streptococcus pyogenes***

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A gene unique to *Streptococcus pyogenes*, called vfr, that negatively regulates speB, an important extracellular protease, has been identified. Disruption of vfr markedly increased SpeB production in a clinical strain of *S. pyogenes* and relieved its growth phase dependency. These findings may provide important insights into the pathogenesis of invasive *S. pyogenes* infections.

Group A streptococcus (GAS) causes many human diseases ranging in severity from milder infections such as pharyngitis to life-threatening necrotizing fasciitis/myonecrosis and streptococcal toxic shock syndrome (reviewed in reference 3). Among the myriad of virulence factors involved in pathogenesis is streptococcal pyrogenic exotoxin B (SpeB), an extracellular cysteine protease that facilitates bacterial survival and dissemination by degrading critical protein components of the host immune system and by modification of bacterial surface proteins (reviewed in reference 29).

We have recently demonstrated that a protease maturation protein gene, prsA, is located immediately downstream of speB-spi (11), that speB-spi and prsA are cotranscribed, and that functional SpeB activity depends on PrsA production (20). In addition, we systematically elucidated the complex transcriptional unit of the polycistronic speB operon (20). Adding to previous reports of two speB transcripts (11, 19, 21), we demonstrated four clearly defined mRNA species hybridizing to the speB-specific probe. The unique characteristics of this operon include two speB promoters separated by 560 bp, two transcriptional terminators separated by the gene prsA, and an independent prsA promoter (20).

Regulation of SpeB gene expression is complex and not fully understood. Neely and Caparon have shown that RopB, an Rgg family member, binds to the speB promoter and is required for speB expression (21). Other transcriptional regulators, including CsrR (also known as CovR) (9), LacD.1 (15), Pel/SagA (14), and Mga (18, 26), have also been implicated in speB regulation; however, direct DNA binding of these regulators to the speB promoter region has not been demonstrated. Finally, some investigators have speculated that additional unknown factors together with RopB control speB gene expression (17).

**Transposon mutagenesis of wild-type GAS.** To further investigate speB regulation, the Tn917 plasmid derivative, pTV1-OK (8), was transformed into a clinical isolate of M-type 3 GAS (strain 88-003) by electroporation. This strain was isolated from a fatal case of streptococcal toxic shock syndrome with necrotizing fasciitis/myonecrosis and was characterized in a previous report (31). Growth of the transformed bacteria, first at 30°C and then at 42°C (8), followed by selection of erythromycin-resistant colonies, yielded mutants with Tn917 transposed to the host chromosome, thereby creating a Tn917-mutagenized library of GAS 88-003. This library was screened for SpeB protease activity by a milk agar plate hydrolysis assay (20). SpeB activity in those with visibly altered protease activity was quantitated by azocasein hydrolysis (7). One of the 700 mutant clones screened, the one named 88-003 transformant #1 (88-003T1) showed a dramatic increase in SpeB protease activity in the stationary-phase (18 h) culture supernatant compared to the wild-type parent strain (mean ± standard deviation, 36.4 ± 0.2 versus 7.2 ± 2.8 μg/ml, respectively); however, no difference in the growth dynamics was observed between the two strains. Furthermore, 88-003T1 produced SpeB during early-log-phase growth (i.e., SpeB levels at 1.5, 3, and 4.5 h were 5.0 ± 0.7, 8.1 ± 1.8, and 15.9 ± 2.2 μg/ml, respectively), at time points when no detectable SpeB activity was produced by the parent strain. In fact, the quantity of SpeB produced by the mutant at 3 h exceeded that produced by the parent strain at 18 h (8.1 ± 1.8 versus 7.2 ± 2.8 μg/ml, respectively).

**Characterization of transposon insertion site.** To characterize the transposon insertion in 88-003T1, a Southern blot of XbaI-digested chromosomal DNA was probed with a 32P-labeled Tn917 DNA fragment probe (Fig. 1A, lower section). Results confirmed a single chromosomal insertion of the transposon in the transformant. The exact location of the Tn917 insertion site in the 88-003T1 chromosome was determined by PCR and sequencing analysis. For this, genomic DNA from the mutant was digested to completion with HindIII and then ligated to a double-strand adaptor (GTACATATTGCGTTAGAACCGCGTAAATACGACTC AATAGGGA) which has a 4-base overhang designed to match HindIII. This material was used as a PCR template in two separate PCRs with two oligonucleotide pairs as indicated in Fig. 1A as Tn917 7047/adaptor and Tn917 5428/adaptor. The resultant PCR products were analyzed by commercial sequencing. A BLASTn search of the returned sequence identified the interrupted gene as SpyM3_0606 in MGAS315, where it was annotated as a hypothetical protein.
tein. We have named this gene vfr, for virulence factor related.

Characterization of the vfr gene. We then PCR cloned a 905-bp DNA fragment from the wild-type strain GAS 88-003 (with vfr1-EcoRI cgggaattcATAGTTAGTTACCAGATT and vfr3-BamHI acgcgtcgacGGACAACTAGTGATTAGGC) and found the sequence of the cloned fragment to be identical to that reported in GenBank for SpyM3_0606 in MGAS315. (Lowercase residues contain EcoRI and BamHI sites, respectively.) This PCR-derived fragment included the entire open reading frame of 747 bp as annotated by GenBank, plus 75 bp upstream of the first ATG, and 85 bp downstream of the stop codon. Within the upstream region, we identified three important and well-conserved elements: a ribosome binding site rich in purines (AAGGAGA) located 5' to the ATG with a 6-bp spacer; a −35 consensus sequence of TTGACG; and a −10 consensus sequence of TAGTAT located 17 bp downstream of the −35 conserved elements.

vfr gene expression in wild-type GAS and the transposon mutant. A vfr-specific probe was derived by PCR using primers vfr2 (ATAGTTAGTTACCAGATT) and vfr4 (ACCATTAGGTGAGTAATCC). With this probe, Northern blotting of RNA isolated from log-phase wild-type GAS strain 88-003 at different stages of growth (3 h, log phase; 5.5 h, early stationary phase; 7 h, late stationary phase). In panels B and C, the intensity of the rRNA bands indicates that relatively equal amounts of RNA were analyzed.
was growth phase dependent, peaking in the early log phase and decreasing as the organism enters the stationary phase (Fig. 1C).

Analysis of SpeB gene expression in the wild type and transposon mutant. Northern blotting with an \textit{speB}-specific probe was performed to determine whether the increased SpeB activity was due to altered transcription or to a posttranscriptional event. Total RNA was purified from the wild-type strain 88-003 and mutant 88-003T1 at early stationary phase (4 h) when SpeB production is initiated. Two micrograms of total RNA from each sample was separated on a 1\% denaturing agarose gel. RNA was transferred by capillary action to a nylon membrane (Hybond-N; Amersham Life Science) with 20\% SSC buffer (1 \times \text{SSC} \text{ is } 0.15 \text{ M NaCl plus } 0.015 \text{ M sodium citrate}). The membrane was air dried and baked at 80\(^\circ\)C for 2 h, prehybridized in Ultrahyb solution (Ambion) at 42\(^\circ\)C for 1 h followed by hybridization for 18 h at 42\(^\circ\)C with the random labeled\(^{32}\)P probe which was an 842-bp internal fragment of \textit{speB} coding region (covering \(\sim 80\%\) of the N-terminal portion) generated by the primer pair \textit{speB-F} (GGTGTCAGATTTGT AAGTCTTTTAGC) and \textit{speB-R} (CGAGAGCTACTCTGCA GAAC). The membrane was washed twice with 2\% SSC and 1\% sodium dodecyl sulfate at 60\(^\circ\)C for 15 min each, and twice with 0.2\% SSC and 1\% sodium dodecyl sulfate at 60\(^\circ\)C for 15 min each then exposed to BioMax light film (Kodak). The results indicated that transcription of \textit{speB} at this stage of growth is weakly detectable from the wild type, but is extremely strong (\(\geq 50\)-fold above wild-type levels) in the transposon mutant (Fig. 2A). Furthermore, transcription of \textit{speB} in 88-003T1 yielded the same four mRNA bands that we have previously shown result from polycistronic transcription of \textit{speB-spi/prsA} (20). These results suggest that \textit{vfr} controls \textit{speB} expression at the level of transcription.

Construction of the \textit{vfr} complementation plasmid. To confirm \textit{vfr}’s role in \textit{speB} regulation, the plasmid Pori::\textit{vfr} was constructed by subcloning the aforementioned 905-bp DNA fragment into EcoRI and BamHI sites of the plasmid Pori23 (28), modified by us to contain \textit{aad9} for spectinomycin resistance inserted between the PstI and SalI restriction sites. The purified \textit{vfr}-containing plasmid, or the empty control plasmid, was then used to electrotransform the \textit{vfr}-deficient mutant strain 88-003T1, and transformants were selected on agar plates containing both erythromycin and spectinomycin. One of the \textit{vfr}-complemented transformants, strain 88-003T1-Comp1 was analyzed further to determine whether the wild-type transcription pattern of \textit{speB-spi/prsA} and production of functional SpeB had been restored. RNA from early-stationary-phase 88-003T1-Comp1 (grown 4.5 h in Todd-Hewitt broth [THB] supplemented with 5 \(\mu\)g/ml erythromycin and 100 \(\mu\)g/ml spectinomycin) was purified, and expression of \textit{speB-spi/prsA} was determined by Northern analysis. In the 88-003T1-Comp1 strain, \textit{speB-spi/prsA} transcription was restored to the low level seen in wild-type GAS 88-003 (Fig. 2B, left) but was unchanged in the 88-003T1 mutant strain transformed with the
empty plasmid (88-003T1-Empty) in Fig. 2B, right). In addition, protease activity in the stationary-phase culture supernatant of the vfr-complemented strain was also restored to wild-type levels, whereas complementation with the empty plasmid had no effect (not shown).

To ensure that the 905-bp fragment contained a functional transcriptional unit for vfr, RNA from pOri::vfr-complemented transformant (88-003T1-Comp1) and from the empty plasmid transformant (88-003T1-Empty) was subjected to Northern analysis using a vfr-specific probe. Expression of vfr was found in the mutant transformed with pOri::vfr (i.e., 88-003T1-Comp1) but was undetectable in the vfr-deficient mutant transformed with the empty rescue plasmid (Fig. 2C).

A Southern analysis was performed to rule out the possibility that the pOri::vfr plasmid had integrated into the genome through homologous recombination. For this, DNA from 88-003T1-Comp1 was purified by the Pitcher method (23), separated on 1% Tris-acetate-EDTA-agarose, and transferred onto a nylon membrane. When the linearized pOri23 plasmid was 32P labeled and used as a probe, the only signal that was detected corresponded to the size of the plasmid and not to that of the higher-molecular-weight genomic DNA (data not shown). This confirmed that vfr was expressed ectopically in this strain.

**Disruption of Vfr in ATCC strain 12384 increases SpeB expression.** To rule out the possibility of a spontaneous mutation in other locations within the genome which might have contributed to the observed changes in speB expression, we utilized a different mutational strategy and a different strain of GAS to confirm our findings. For this, we chose GAS strain 12384 from the American Type Culture Collection (ATCC). This strain is an M-type 3 strain (12) that produces many of the exotoxins known to contribute to pathogenesis, including SpeB, and is lethal in a murine model of severe GAS myonecrosis (30, 32). An EcoRV genomic DNA fragment covering the middle section of the vfr coding region (Fig. 1) was cloned from ATCC 12384 and then subcloned into plasmid pJRS233’s Smal site (22) to generate pJRS233vfrRV. The purified plasmid pJRS233vfrRV was then transformed into the ATCC 12384 strain. Transformants were selected on THB agar plates containing erythromycin, and a selected colony was cultured at 30°C overnight in THB medium with 10 μg/ml erythromycin (THB-Ery). The overnight culture was diluted in fresh THB-Ery and cultured at 39°C until the stationary phase was reached. This was repeated for three rounds of culture followed by plating on THB agar with erythromycin. Southern analysis of the resulting clone again verified a single plasmid insertion (data not shown). Northern analysis of this clone again verified a single plasmid insertion. This was repeated for three rounds of culture followed by plating on THB agar with erythromycin. Southern analysis of the resulting clone again verified a single plasmid insertion (data not shown). Northern analysis of this clone again verified a single plasmid insertion.

**Ruling out polar effects.** As shown in Fig. 1A, vfr is located in a gene cluster surrounded by the clpX/GTPase and clpL genes, which code for ATP-binding subunits of ClpP, an ATP-dependent caseinolytic protease involved in energy-dependent proteolysis in some prokaryotes (27). Currently, information about the role of such proteases in *Streptococcus pyogenes* is lacking. The open reading frames for clpX and the GTPase gene are only 10 bp apart, indicating they might be transcribed together. vfr is 757 bp downstream of the clpX coding region and 148 bp downstream of the GTPase coding region. Tn917 insertion into vfr could potentially create a polar effect that disturbs expression and function of clpX. To address this issue, we performed a Northern analysis to determine whether clpX’s expression was changed in the vfr-deficient transposon mutant compared to the wild-type strain. At 4 h, clpX was not detectable in either strain by a 32P-labeled clpX-specific probe (a PCR-derived probe from wild type GAS using the primer pair clpX-s [ATGGCAGGAAATGGAAC] and clpX-a [TTAACG TGTCCTAANAACGTTG] and covering the entire clpX open reading frame), even after an extended period of exposure of the X-ray film (data not shown). This suggests that clpX is not likely a positive regulator of speB and that a polar effect on clpX from the vfr-Tn917 insertion can be largely, but not completely, ruled out as a cause for increased speB expression in the transposon mutant.

Furthermore, clpL, which is located downstream of vfr (although read in the opposite direction) may substitute for clpX. RNA analysis of clpL with a gene-specific probe covering 646 bp of the C-terminal portion of the clpL open reading frame (with primers clpL-s [CGATCGCACTGCTGTGTCAAAAT TG] and clpL-a [TTACTGATCGTCTTTTGC]) indicates that clpL is expressed equally at 4 h in all of the strains tested, (i.e., wild-type 88-003, the vfr-deficient transposon mutant 88-003T1, and the vfr-complemented strain 88-003T1-Comp1) (Fig. 3). Together, these results suggest that insertion of the transposon into vfr did not alter expression of nearby genes.

**Vfr’s distribution.** Northern analysis detected vfr mRNA in GAS of M-types 1, 3, 5, and 28, but not in group B streptococcus or *Staphylococcus aureus* (data not shown). BLAST searching of the vfr nucleotide sequence (NCBI BLASTn 2.2.17 [1] with the expected value set at 10) resulted only in matches to hypothetical proteins found in the 13 completed *S. pyogenes* genomes. Furthermore, BLAST searching of Vfr’s predicted amino acid sequence among the 788 available prokaryotic genomes (NCBI BLASTp 2.2.17 [1] with the expected...
value set at 10) revealed that only proteins found in *S. pyogenes* matched with 99 to 100% identity. However, proteins with low-level homology (25%) to different portions of the Vfr sequence were found in other species, although a common function was not readily discernible among these widely varied protein regions. Thus, *vfr* appears to be unique to *S. pyogenes*.

Considerable evidence suggests that toxin gene expression in many clinically important bacteria is coordinately regulated. For GAS, logarithmic growth is associated with maximal expression of proteins involved in nutrient uptake, cell wall synthesis, and bacterial adherence and colonization. Entrance of GAS into the stationary phase of growth is associated with decreased surface expression of M-protein, hyaluronic acid capsule (33), and the penicillin binding proteins (32) and increased production of some extracellular toxins, including SpeB (21). More than a dozen possible two-component systems have been identified in GAS, the majority of which have not been fully characterized (reviewed in reference 10). Some of these (e.g., *csrRS covRS*) are known to control SpeB production. In addition, some of the known standalone SpeB regulators characterized thus far could be associated with as yet unidentified transcriptional activators or repressors or other sensory elements. Thus, mechanisms controlling expression and maturation of SpeB, and their links to the growth cycle and nutrient availability, are highly complex and an open field waiting to be fully elucidated.

The present study clearly demonstrates that a previously uncharacterized hypothetical gene unique to *S. pyogenes*, which we call *vfr*, negatively regulates SpeB. Furthermore, disruption of this gene appeared to relieve the known growth phase dependency of SpeB production. If confirmed by additional studies, our results suggest that Vfr may mimic or augment other global regulators that have demonstrated at least some element of growth phase dependency (e.g., Mga, CsrRS, Rgg, and FasBCAX [6]). For example, proteins positively regulated by Mga are typically cell associated and maximally expressed during log-phase growth. This system also regulates the oligopeptide and dipeptide permease operons (*opp* and *dpp*, respectively), the inactivation of which diminishes *speB* mRNA levels (24, 25). Similarly, the global regulator, Rgg, influences exoprotein production as the organism shifts from log to stationary phase (4) and is required for SpeB production (5); however, ectopic expression of RopB does not alter the temporal dynamics of SpeB production (21). In contrast, interruption of *fasBCAX* prolongs expression of surface adhesins and suppresses extracellular toxin production well into the stationary phase (13). A possible reciprocal interaction between the Fas system and *vfr* is an intriguing notion. Recently Loughman and Caparon (15, 16) have identified LacD.1 as a global regulator of virulence factor expression in *S. pyogenes* and that disruption of *lacD.1* uncouples SpeB production from its growth phase dependency. Whether *vfr* and LacD.1 may interact is another interesting topic under investigation in our laboratory.

The mechanism by which *vfr* negatively regulates SpeB production remains to be elucidated. Vfr may act as an independent transcription regulator or may interact with other known transcriptional regulators such as RopB. However, based on the predicted amino acid sequence, it is unlikely that Vfr is a DNA binding protein, although this possibility cannot be totally discounted. Further analysis of *vfr*’s predicted amino acid sequence with an internet computer program (http://www.predictprotein.org) revealed an N-terminal helical transmembrane region, suggesting Vfr may be a membrane-associated protein. If so, Vfr may function as a regulatory sensor and thus may be part of a novel two-component system. However, analysis with the SignalP 3.0 algorithm (2) revealed a strong N-terminal 37-amino-acid signal peptide with a predicted LFG signal peptidase cleavage site, suggesting Vfr may be a secreted protein. Definitive localization studies are currently ongoing in our laboratory.

In summary, we have identified a novel gene unique to *S. pyogenes* we call *vfr* that negatively regulates expression of the virulence factor SpeB. Further characterization of *vfr* is under way and may resolve unanswered questions regarding the global toxin regulatory network in *S. pyogenes*. An understanding of the hierarchy and integration of these various regulatory mechanisms could help explain the ability of the organism to cause diverse infections and may offer new therapeutic targets for prevention or treatment.

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None of the authors of this work has any conflict of interest to declare.

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