Eubacterial SpoVG homologs constitute a new family of site-specific DNA-binding proteins

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Eubacterial SpoVG Homologs Constitute a New Family of Site-Specific DNA-Binding Proteins

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Abstract

A site-specific DNA-binding protein was purified from Borellia burgdorferi cytoplasmic extracts, and determined to be a member of the highly conserved SpoVG family. This is the first time a function has been attributed to any of these ubiquitous bacterial proteins. Further investigations into SpoVG orthologs indicated that the Staphylococcus aureus protein also binds DNA, but interacts preferentially with a distinct nucleic acid sequence. Site-directed mutagenesis and domain swapping between the S. aureus and B. burgdorferi proteins identified that a 6-residue stretch of the SpoVG α-helix contributes to DNA sequence specificity. Two additional, highly conserved amino acid residues on an adjacent β-sheet are essential for DNA-binding, apparently by contacts with the DNA phosphate backbone. Results of these studies thus identified a novel family of bacterial DNA-binding proteins, developed a model of SpoVG-DNA interactions, and provide direction for future functional studies on these wide-spread proteins.

Introduction

To be successful, single-celled organisms must efficiently and rapidly adapt to changing conditions. This is often accomplished through exquisite regulatory networks involving numerous, dynamic trans-acting factors. Prokaryotic proteins that bind to nucleic acids govern virtually every cellular process, including nucleoid organization, transcription, translation, and DNA replication, modification, repair, and recombination. Remarkably, most DNA-binding proteins are poorly characterized, and it is likely that many more await discovery.

In our studies of the VlsE antigenic variation system of Borellia burgdorferi, the causative agent of Lyme disease [1,2], we discovered that these bacteria produce a cytoplasmic protein which specifically binds a DNA site within the vlsE open reading frame. Using a powerful, unbiased approach, we identified that protein to be the borrelial SpoVG. A broad range of Eubacteria, including many important human pathogens, encode homologs of SpoVG. The name derives from observations that Bacillus spp. spoVG mutants are unable to complete stage five of sporulation [3–5]. Bacillus spp. mutants exhibit additional defects, such as abnormal cell cycle and division [4,6,7]. Staphylococcus aureus spoVG mutants are less virulent than are wild-type bacteria, and produce significantly lower levels of several pathogenesis-related factors [8–10]. Many organisms, production of spoVG is developmentally regulated and often utilizes alternative sigma factors [11–23]. The three dimensional structures have been determined for SpoVG from S. aureus and other species, and found to be very highly conserved ([24], and Protein Data Base [PDB] accession numbers 2IA9, 2IA9X, 2IA9Z). However, until our discovery, the biochemistry of SpoVG remained a mystery.

Here we demonstrate that the SpoVG homologues of Borellia burgdorferi, Staphylococcus aureus, and Listeria monocytogenes all bind to DNA. Further investigations determined that, while SpoVG members are highly similar, they have evolved to bind different consensus sequences. Alanine mutagenesis and domain shuffling revealed residues and microdomains required for generalized DNA binding and for nucleotide sequence specificity.

Results

Identification of B. burgdorferi SpoVG as a Site-specific DNA-binding Protein

As part of our studies of the vlsE system, we postulated that B. burgdorferi expresses a cytoplasmic factor(s) that binds near the recombination site, to help control genetic rearrangement. Addressing that hypothesis, we observed that incubating cell-free B. burgdorferi cytoplasmic extract with vlsE DNA retarded the electrophoretic mobility of DNA, consistent with a DNA-protein complex (Fig. 1). This complex was not evident when cytoplasmic
extracts were heat denatured or treated with proteinase K, indicating the need for a properly folded, intact protein (data not shown). Additional EMSAs narrowed the protein-binding site even further. The 70 bp labeled probe F27B-R10 bound the cytoplasmic protein, and binding was competed by the unlabeled version of that DNA sequence, fragment F27–R10 (Fig. 1A and B, lane 4). DNAs flanking those 70 bp did not compete for protein binding (Fig. 1B, lanes 3 and 5). These results indicate that the borrelial protein binds a DNA sequence of approximately 70 bp (X on Fig. 1A), and that neither of the repeat regions flanking the recombination site is involved in protein binding.

To identify the unknown factor, we took advantage of a DNA affinity chromatography method developed in our laboratory, which has identified several other novel sequence-specific DNA-binding proteins [25–27]. Using a segment of vlsE that included the high-affinity binding site as bait, a protein of approximately 12 kDa was purified. Buffers containing at least 500 mM NaCl were required to elute the protein off the DNA, indicating that the trans acting factor had a high affinity for vlsE bait DNA (Fig. 2). Matrix assisted laser desorption- time of flight (MALDI-TOF) MS/MS analysis identified the protein as being encoded by open reading frame BB0785, a hypothetical protein of unknown function, with a corresponding Mascot score of 212. Control reactions that used the same cell-free extracts and different DNA baits did not pull-down this protein (data not shown).

Due to its homology with the SpoVG proteins of many bacterial species, we have retained that name for the borrelial gene and protein (Fig. 3). To confirm that this protein was responsible for the protein-DNA complex formed by cytoplasmic extracts, we purified recombinant B. burgdorferi SpoVG (SpoVG<sub>Bb</sub>) and repeated the EMSAs. Indeed, recombinant SpoVG<sub>Bb</sub> bound to probe F27B-R10 (Fig. 4A). This 70-mer was dissected and one 18 bp fragment was found to be required and sufficient for SpoVG-binding (Figs. 4B & C). SpoVG<sub>Bb</sub> bound to its high-affinity target DNA with an apparent disassociation constant (K<sub>D</sub>) of 308 (±31) nM. Further controls incorporated erp Operator DNA, a region of DNA known to be bound by other B. burgdorferi DNA-binding proteins [25,28,29]; SpoVG<sub>Bb</sub> failed to bind this sequence, confirming its specificity for vlsE DNA (Fig. 4D). The identified SpoVG<sub>Bb</sub>-binding sequence does not occur anywhere else in the B. burgdorferi genome, although it is possible that this protein may bind sequences that differ slightly from the site within vlsE. These studies were the first to demonstrate a function for a SpoVG orthologue. The role(s) of SpoVG<sub>Bb</sub> in vlsE rearrangement is still under investigation, and is beyond the scope of this communication on the biochemistry of SpoVG-DNA interactions.

**S. aureus SpoVG is also a Site-specific DNA-binding Protein**

Bioinformatics indicate that many spore and non-spore forming bacteria, Gram positive and Gram negative, encode a SpoVG protein (Fig. 3). Given the high degree of sequence conservation, we hypothesized that these orthologues also bind DNA. Compared to wild-type bacteria, S. aureus spoVG mutants express significantly less capA-H mRNA and synthesize reduced levels of capsule [9,17]. We hypothesized that S. aureus SpoVG (SpoVG<sub>sa</sub>) might bind DNA near the cap operon promoter. This was confirmed by EMSA, which demonstrated that recombinant SpoVG<sub>sa</sub> bound to S. aureus (Newman) cap5 5’-non-coding DNA in a dose dependent fashion (Fig. 5, lanes 2–4). Heat denaturation or proteinase K treatment eliminated the shifted EMSA band, confirming that this complex contained functional protein (Fig. 5, lanes 11 and 12). In order to determine the relative affinity of the SpoVG<sub>sa</sub>-DNA interaction, three independent protein preparations and multiple EMSAs with labeled cap probe were performed with saturating

![Figure 1. A B. burgdorferi cytoplasmic protein binds DNA within the vlsE open reading frame. (A) Schematic of the vlsE expression locus and DNAs uses for EMSA. In the upper illustration of the vlsE ORF, the gray areas represent the invariable regions, the white area represents the hypervariable region, and the black bars indicate the direct repeat sequences. Below that, the location of each labeled or unlabeled EMSA DNA is represented by thick or thin black horizontal lines, respectively. “DR” indicates the directly-repeated sequences bordering the variable region of vlsE. (B) EMSAs using B. burgdorferi cytoplasmic extracts. Lane 1–6:1 nM of labeled probe F27B-R10. Lane 2–6:10 μg cell-free cytoplasmic extract. Lane 3:100-fold molar excess unlabeled competitor F35-R14. Lane 4:100-fold molar excess unlabeled competitor F27–R10. Lane 5:100-fold molar excess unlabeled competitor F29–R16. Lane 6:100-fold molar excess unlabeled competitor F33–R22. doi:10.1371/journal.pone.0066683.g001](image)

![Figure 2. DNA-affinity chromatographically purified SpoVG<sub>Bb</sub>-vlsE probe affixed to magnetic beads was incubated with cell-free extracts and bound protein were eluted by titration with increasing concentrations of NaCl. Eluates were separated by SDS PAGE, stained with Sypro Ruby. M. Molecular mass standards. Lanes 1–3. Proteins eluted with 500, 750, and 1000 mM NaCl elution, respectively. doi:10.1371/journal.pone.0066683.g002](image)
concentrations of SpoVGsa. These experiments indicated an average $K_D$ of 316 (±42) nM.

In a whole transcriptome screen of a $S. aureus$ spoVG mutant, significant alterations in several other virulence-related loci were documented, including fmtB, esxA, and lukED [9]. The ability of SpoVGsa to bind near the promoters of those genes was evaluated using each DNA as an unlabeled EMSA competitor against labeled cap5 DNA. The fmtB, esxA, and lukED 5’-non-coding DNAs each competed with labeled cap5 probe for binding of SpoVGsa (Fig. 5, lanes 5–7). Control studies using unlabeled competitors derived from the esxA or cap5A open reading frames had substantially lesser effects on SpoVGsa binding to the labeled cap5 probe (Fig. 5, lanes 9 and 10). These results indicate that the 5’-non-coding regions of cap5, fmtB, esxA, and lukED all contain a unique sequence(s) to which SpoVGsa binds with high affinity and specificity.

Additional EMSAs using a smaller probe and unlabeled competitors narrowed down the high-affinity SpoVGsa-binding sequence in cap5 promoter-proximal DNA. Probe cap41 contains a SpoVG-binding site (Fig. 6, lane 2). Three unlabeled 28 bp DNAs, which span the 62 bp sequence of probe cap41, were included in EMSAs at molar excesses over probe cap41. This type of analysis prevents a possible bias towards probe and/or competitor length, while controlling for potential high affinity interactions at the ends of the probe. At a constant concentration of SpoVGsa, addition of competitor A decreased the amount of bound probe and increased the amount of free DNA (Fig. 6, lanes 3–5). In contrast, 5-fold greater concentrations of competitors B or C did not detectably affect SpoVGsa binding to probe cap41 (Fig. 6, lanes 6 and 7). These data indicate that the high affinity-binding site is contained within the 28 nucleotides of competitor A.

MEME (Multiple Em for Motif Elicitation) analyses of the DNAs bound by SpoVGsa indicated that all contain at least two 5-TAATT\textsuperscript{1/3}-TAATT\textsuperscript{1/3} sequences (Fig. 7A). Competitor A contains two copies of that motif. To evaluate whether this motif is involved with SpoVG binding, a competitor with mutated motifs was incorporated into subsequent EMSAs (Fig. 7C). SpoVGsa exhibited greater than five-fold higher affinity for the wild-type competitor over the mutant (Fig 7B). Taken together, these results demonstrate that the $S. aureus$ SpoVG protein preferentially binds to DNA containing a TAATT\textsuperscript{1/3}A motif. Whether SpoVGsa will bind to any such sequence or if surrounding DNA sequences/structures contribute to protein binding remains to be determined.

**Different SpoVG Homologues Bind to Different DNA Sequences**

The vlsE probe, to which SpoVGsa binds with high-affinity and specificity, does not possess the SpoVGsa consensus binding motif. These observations suggested that SpoVG homologues might bind to divergent, distinct DNA sequences. With this in mind, we incubated equal concentrations of SpoVGsa or SpoVGsb with labeled vlsE and cap41 probes in independent EMSAs. SpoVGsb bound to the vlsE probe, but not cap41 (Fig. 8A). Likewise, SpoVGsa bound to only the cap41 probe (Fig. 8B).

To further address our hypothesis that SpoVG homologues act in a similar fashion, but interact with different sequences, we purified the SpoVG homologue from another firmicute, *Listeria monocytogenes* (SpoVGLo). SpoVGLo bound $S. aureus$ cap41 promoter DNA but not $B. burgdorferi$ vlsE DNA (Fig. 8C).

**Chimeric SpoVG Proteins Identify Residues Involved with Sequence Specificity**

Orthologous proteins are under selective pressure to maintain function, but can diverge in amino acid composition to accommodate the needs of the individual species. Protein structural predictions indicated that SpoVG homologues possess a hypervariable alpha helix at the carboxy terminus (Fig. 3). We suspected that it was this variable domain that contributed to the above-described DNA sequence specificity. To address this hypothesis, we created two different chimeric SpoVG proteins. The staphylococcal SpoVG protein was mutated at residues $S^{66}$ through $E^{71}$ and changed to the corresponding borellial SpoVG residues, creating the chimeric variant SpoVGsb-Lo. We reciprocated this strategy by exchanging residues $Q^{13}$ through $A^{19}$ of SpoVGsb with those of the $S. aureus$ protein, generating the chimeric protein SpoVGsa-Lo (Fig 8AB and Fig. 3). For both chimeras, exchanging 6 residues was sufficient to permit binding to the alternative consensus sequence. SpoVGsb-Lo bound to the cap41 probe, but could no longer bind to the vlsE probe. The SpoVGsa-Lo protein now bound vlsE DNA. That chimera retained

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**Figure 3.** SpoVG is a highly conserved Eubacteria protein. Illustrated is an alignment of the predicted sequences of SpoVG proteins from 19 different families of 6 different phyla. Alignment was performed using Geneious software, Pfam 200, with 1000 iterations. Identical or homologous amino acids are indicated by same-colored boxes. Note that these analyses grouped the SpoVG protein of the opportunistic oral pathogen *Prevotella denalis* with the Gram-positive *Bacilli* class, although it is currently considered to be a member of the Bacteroides. Consistent with these results, *P. denalis* has morphological and biochemical features which differ from other species in the genus *Prevotella* and class *Bacteroides* [51]. Red arrows indicate residues demonstrated to be involved in SpoVG-DNA interactions. Green asterisks denote conserved residues that were found to be not required for binding DNA. The magenta box indicates residues of SpoVG that were found to be conserved.

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a slight ability to interact with the cap41 DNA, albeit at a dramatically reduced affinity for which a K_d could not be calculated (Fig. 8B). Taken together, these results demonstrate that sequence divergence within the alpha helix contributes to DNA sequence specificity.

Conserved Residues Essential for DNA-protein Complexes

Bacterial proteins that perform analogous functions often retain similar biochemical and structural features in order to interact with their respective ligands [30]. We reasoned that, since three different SpoVG proteins interact with DNA, conserved residues common to all SpoVG orthologues might be required for non-specific substrate binding. Recombinant SpoVG_Sa and SpoVG_Bb proteins were produced that included single or double amino acid substitutions at conserved positions (Fig. 3, and Table 1). These mutant proteins were tested for their abilities to interact with their respective high-affinity DNA sequences.

Initial investigations targeted a doublet of positively charged residues (R and K), which were conserved in all SpoVG homologues (Fig. 3). The two charged residues are predicted to project inward from an abbreviated β-sheet, toward the carboxy-terminal alpha helix. Alanine substitutions at position R53–R54 of SpoVG_Bb or K50–R51 of SpoVG_Sa impaired DNA binding. Addition of mutant proteins at five-fold excess over the dissociation constant of the wild-type protein still did not produce a detectable EMSA shift (Figs. 3 and 9). To assay residues independently, SpoVG_Sa K50A and SpoVG_Sa R51A were created. These variants exhibited the same deficiency in DNA binding as

Figure 4. Identification of a SpoVG_Sa high-affinity binding site. Recombinant SpoVG_Sa binds specifically to an 18 bp sequence of the vhs open reading frame. (A) Lanes 1–3: 1 nM of labeled F27B-R10. Lane 2: 300 nM SpoVG_Sa. Lane 3: 600 nM SpoVG_Sa. (B) Lanes 1–3: 1 nM of labeled F77B-R10. Lane 2: 300 nM SpoVG_Sa. Lane 3: 600 nM SpoVG_Sa. (C) Lanes 1–3: 1 nM of labeled F27B-R4. Lane 2: 300 nM SpoVG_Sa. Lane 3: 600 nM SpoVG_Sa. (D) Lanes 1–3: 1 nM of labeled erp operator DNA. Lane 2: 300 nM SpoVG_Sa. Lane 3: 600 nM SpoVG_Sa. doi:10.1371/journal.pone.0066683.g004
did the double mutant, confirming that both conserved residues are required for DNA binding. Mutations to other conserved, positively charged residues did not have any significant effects on DNA binding (Fig. 3, Table 1, and data not shown). Additionally, none of the other mutant proteins exhibited altered sequence preference (data not shown).

Site-directed Mutagenesis did not Affect Multimerization

Replacing charged or polar residues with a small, non-polar, uncharged alanine can interfere with protein-protein interactions, or cause protein misfolding [31]. To that end, sizing chromatography and tandem native/denaturing PAGE analysis were used to examine the native state of SpoVG<sub>sa</sub>. The recombinant protein has a molecular mass of 14.6 kDa. By two independent methods, our data indicate that SpoVG<sub>sa</sub> forms a 55–60 kDa complex in solution, consistent with a tetramer (Fig. 10). The complexes disappeared when samples were denatured, demonstrating that these bands were not the results of contamination (Fig. 10C). None of the SpoVG<sub>sa</sub> mutants exhibited diminished multimer formation, suggesting that the mutants which were impaired for DNA binding still retained their ability to fold correctly and form higher ordered species in solution.

Discussion

The current studies yielded several novel findings that impact a broad range of Eubacterial species. First, SpoVG orthologues from three distinct bacteria bound DNA. For several bacterial species, it is known that these small proteins play key roles in critical cellular processes, which we can now hypothesize are due to SpoVG-DNA interactions. Second, these discoveries help explain why SpoVG was found in association with the <i>S. aureus</i> nucleoid, and the involvement of the <i>B. subtilis</i> orthologue with nucleoid organization [4,32]. Third, while SpoVG proteins are highly conserved overall, the <i>S. aureus</i> and <i>B. burgdorferi</i> proteins interact

Figure 5. SpoVG<sub>sa</sub> binds specifically to <i>S. aureus</i> cap5, fmtB, lukED, and saeX DNAs adjacent to the promoter. Illustrated are EMSAs with <i>S. aureus</i> SpoVG<sub>sa</sub> labeled cap5 5’ non-coding DNA, and various unlabeled competitor DNAs. Lanes 1–12: 5 nM of labeled capA promoter DNA. Lanes 2–4: Increasing amounts of SpoVG<sub>sa</sub> (0.2 μg, 0.4 μg, and 0.6 μg, respectively). Lanes 5–12: 0.8 μg of SpoVG<sub>sa</sub>. Lanes 5–8: 50-fold molar excess of unlabeled fmtB, lukED, exsA, or cap5 5’ non-coding DNAs, respectively. Lane 9: 50-fold molar excess of exsA ORF DNA. Lane 10: 50-fold molar excess of cap5A ORF DNA. Lane 11: SpoVG<sub>sa</sub> was heated to 99°C for 5 minutes before use in EMSA. Lane 12: SpoVG<sub>sa</sub> was preincubated with 5 mg/ml of Proteinase K before use in EMSA.

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Figure 6. SpoVG<sub>sa</sub> interacts specifically with a 28 bp region adjacent to the cap5 promoter. EMSA with a labeled 62 bp probe derived from of cap5 5'-non-coding DNA (cap41) and different concentration of 28 bp cold competitors. All lanes contain 5 nM labeled cap41 DNA. Lanes 2–6 also include 0.9 μg of SpoVG<sub>sa</sub>. Lanes 3 and 4: 25 fold molar excess competitor A or mutant competitor A, respectively. Lanes 5 and 6: 50 fold molar excess of competitor A and mutant competitor A, respectively. Lane 7: 50 fold molar excess of competitor B. Lane 8: 50 fold molar excess of competitor C.

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Figure 7. The palindromic motif 5'-ATTAA<sub>A</sub>/A-3' is required for SpoVG<sub>sa</sub> binding. (A) The conserved sequence, 5’ to 3’, identified by multiple motif analysis of promoters bound to and influenced by SpoVG<sub>sa</sub>. (B) EMSA with a labeled probe cap41, derived from cap5 sequence, and two different 28 bp cold competitors. All lanes contain 5 nM labeled cap41 DNA. Lanes 2–6 also include 0.9 μg of SpoVG<sub>sa</sub>. Lanes 3 and 4: 25 fold molar excess competitor A or mutant competitor A, respectively. Lanes 5 and 6: 50 fold molar excess of competitor A and mutant competitor A, respectively. (C) Sequences of probe cap41 and competitors. The differences between the wild type and mutant competitors are indicated by boldface italics.

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preferentially with distinct DNA sequences. Given the amino acid divergence among different orthologs’ carboxy-terminal alpha helices, we speculate that this feature may also be true for other SpoVG homologues. Finally, we identified two residues, whose biochemical properties are conserved among SpoVGs, that are essential for DNA interactions.

Residues involved with maintaining SpoVG secondary structure model well between species, suggesting that the solved crystal structures are likely to be representative of all orthologous proteins. Merging all of these data, we propose a model for SpoVG binding (Fig. 11). Solvent-accessible, positively charged residues are located adjacent to the alpha helix and can stabilize duplex binding through electrostatic interactions with the phosphate backbone of DNA. These are residues R53 and R54 of SpoVG\textsubscript{Bb}, and K50 and R51 of SpoVG\textsubscript{Sa}. Those residues extend into a pocket, while the alpha helix is arranged perpendicularly to provide base-edge specificity through interactions by residues extending into the pocket (Figs. 10 and 11). Notably, the \textit{B. burgdorferi} and \textit{S. aureus} alpha helices are out of phase by approximately one turn of the alpha helix, presenting residues with dissimilar hydrogen-donating and hydrogen-accepting capabilities on the upper helical face ([31], and Fig. 11). Independent evolution of the two studied SpoVG proteins resulted in different nucleic acid binding specificity. Our data suggest that SpoVG homologs of different bacterial species may bind to distinct DNA sequences, and possibly exert different effects on physiology. Similar phenomena have been documented that alter the specificity, diversify the signal, and eliminate unwanted cross-talk between sensor histidine kinases and response regulators in two-component signal transduction systems [33–35].

The mechanisms by which \textit{S. aureus} controls of virulence-associated genes are poorly understood. The identification of a SpoVG\textsubscript{Sa}-binding site adjacent to the \textit{cap} promoter suggests that SpoVG\textsubscript{Sa} may play a direct role in controlling capsule production. Indeed, \textit{cap} transcription is significantly reduced in \textit{spoVG}\textsubscript{Sa} mutant \textit{S. aureus} [9], and \textit{S. aureus} lacking a SpoVG\textsubscript{Sa}-binding site in the \textit{cap} promoter exhibit reduced \textit{cap} transcription [36]. However, expression of the \textit{cap} operon has been reported to be controlled by at least 12 other regulatory factors [9,17,36–40]. Studies are currently under way to define binding-sites of the other regulatory factors, and to understand the ways in which these many regulators interact with each other and with RNA polymerase to control \textit{cap} expression.

The role of SpoVG\textsubscript{Bb} in \textit{B. burgdorferi} \textit{vlsE} genetic rearrangement remains to be determined. The specialized recombination processes involved are complex and highly regulated, occurring only during mammalian infection but never during tick colonization or in culture [2,41]. Recombination of \textit{vlsE} is RecA independent, requires holiday junction resolvases, and may involve G-quadruplex DNA [42–44]. Our preliminary studies suggest that SpoVG may interact with other, as-yet unidentified factors. We are continuing studies to identify other players in the
Bacterial SpoVG DNA-Binding Proteins

Table 1. Plasmids used in this study.

| Plasmid Name | Backbone | Organism/Target locus | Description |
|--------------|----------|-----------------------|-------------|
| pBLJ340     | pET101   | L.monocytogenes/spoVG | rSpoVG      |
| pBLJ117     | pET101   | B.burgdorferi/spoVG  | rSpoVG      |
| pBLJ341     | pET101   | B.burgdorferi/spoVG  | ΔSpoVG R53A, R54A |
| pBLJ342     | pET101   | B.burgdorferi/spoVG  | ΔSpoVG H65A  |
| pBLJ343     | pET101   | B.burgdorferi/spoVG  | ΔSpoVG R72A  |
| pBLJ347     | pET101   | B.burgdorferi/spoVG  | ΔSpoVG BiQDFRKA Sa SDMRQE |
| pBLJ505     | pET101   | S.aureus/spoVG       |             |
| pBLJ351     | pET101   | S.aureus/spoVG       | ΔSpoVG K50A, R51A |
| pBLJ352     | pET101   | S.aureus/spoVG       | ΔSpoVG H62A  |
| pBLJ353     | pET101   | S.aureus/spoVG       | ΔSpoVG R69A  |
| pBLJ354     | pET101   | S.aureus/spoVG       | ΔSpoVG K50A  |
| pBLJ355     | pET101   | S.aureus/spoVG       | ΔSpoVG R51A  |
| pBLJ357     | pET101   | S.aureus/spoVG       | ΔSpoVG SaSDMRQE BbQDFRKA |
| pBLJ506     | pCR2.1   | S.aureus/cap5A       | cap5’ UTR   |
| pBLJ507     | pCR2.1   | S.aureus/fmt         | fmtB UTR    |
| pBLJ508     | pCR2.1   | S.aureus/esxA        | esxA UTR    |
| pBLJ509     | pCR2.1   | S.aureus/luk         | lukED UTR   |
| pTB7a       | pCR2.1   | B. burgdorferi/vlsE  | vlsE – 80 +240 |

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Figure 9. Alanine mutagenesis determined residues required for binding DNA. Lanes 1, 2, 5 and 6 contain 2 nM labeled vlsE probe. Lanes 3, 4, and 7–12 contain 2 nM labeled cap41 probe. Additional ingredients of each EMSA are: Lane 2, 1.5 μM mutant SpoVGsa R53A-R54A; Lane 4, 1.5 μM mutant SpoVGsa K50A-R51A; Lane 6, 500 nM wild-type SpoVGsa; Lane 8, 1.5 μM mutant SpoVGsa K50A; Lane 10, 1.5 μM mutant SpoVGsa R51A; Lane 12, 500 μM wild-type SpoVGsa.
doi:10.1371/journal.pone.0066683.g009

Figure 10. Site directed mutagenesis did not influence SpoVG oligomerization. Results of panels A through D illustrate HPLC sizing column chromatography of wild type and mutant SpoVGsa proteins. For some preparations, proteins eluted with a retention time of approximately 7 minutes, which corresponds with a molecular mass >440 kDa and are composed of protein aggregates. (A) Wild-type SpoVGsa; (B) SpoVGsa K50A-R51A; (C) SpoVGsa K50A; (D) SpoVGsa R51A. Peaks marked with red asterisks indicate retention volumes corresponding with approximately 55–60 kDa. Panels E and F illustrate proteins separated following native of denaturing PAGE, respectively. M, Molecular mass standards; Lane 1, 1.5 μM mutant SpoVGsa K50A-R51A; Lane 2, 1 μM mutant SpoVGsa K50A; Lane 3, 1 μM mutant SpoVGsa R51A; Lane 4, 1 μM wild-type SpoVGsa.
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In these studies, those of the firmicute S. aureus and the spirochete B. burgdorferi, each bound with high affinities to distinct DNA sequences. These data suggest that, despite the overall similarities between SpoVG homologs of different species, each may preferentially bind to a different DNA sequence. The amino acid sequence of the SpoVG α-helix was found to be critical for DNA sequence-specificity. Two addition, invariant residues are essential for DNA-binding, probably through contacts with the negatively-charged DNA backbone. These results provide a framework upon which to define the roles of the ubiquitous SpoVG proteins in bacterial pathogenesis and cellular physiology.

Materials and Methods

Bacterial Strains

S. aureus strain Newman was cultured at 37°C in Luria Bertani (LB) broth with agitation. B. burgdorferi strain B31 was propagated in Barbour-Stoenner-Kelly (BSK)-II broth at 34°C [45]. Whole genomic DNAs from B. burgdorferi and S. aureus were purified using Qiagen genomic DNA extraction kits, following the manufacturer’s recommend procedure (Valencia, CA). Purified L. monocytogenes strain EGD-e genomic DNA was a gift from Dr. Sarah D’Orazio.
DNA-affinity Chromatography

A protein was purified from B. burgdorferi cytoplasmic extract based on its affinity for vlsE DNA bait, using previously-described procedures [25,27]. Bait DNA was generated by PCR of the B. burgdorferi vlsE coding region using one 5'-biotin-modified and one unmodified oligonucleotide (Table 2). A single band that eluted in buffer containing 750 mM NaCl was excised and MALDI-TOF

Figure 11. Monomeric structures of SpoVG$_{Sa}$ and SpoVG$_{Bb}$, modeled on the solved S. epidermidis SpoVG protein structure. Residues required for DNA-binding and those involved with sequence specificity are indicated, with different colors reflecting biochemical properties of amino acids: Gray = positively charged, Red = negatively charged, and Green = polar, uncharged. (A) SpoVG$_{Sa}$ (B) SpoVG$_{Bb}$.

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Table 2. Oligonucleotides used in this study.

| Name       | Sequence (5’3’) | Target     | Modification |
|------------|----------------|------------|--------------|
| Bio CapUp F-1 | CTA TCT GAT AAT AAT CAT CTA ACT CAC | capA5 5’UTR | 5’ Biotin   |
| CapUp F-2   | TAT TTA CCT CCC TTA AAA ATT TTC    | capA5 5’UTR | None        |
| CapUp F-1   | CTA TCT GAT AAT CAT CTA ACT CAC    | capA5 5’UTR | None        |
| fmtUp F-1   | GTC ATC CTC CTG GTT GAT TAT TC     | fmtB 5’UTR  | None        |
| fmtUp R-2   |                                  | fmtB 5’UTR  | None        |
| lukEUp F-1  | CTT AAA CAT AAG TTC CAC TTC CTTC   | lukE 5’UTR  | None        |
| lukEUp R-2  |                                  | lukE 5’UTR  | None        |
| esxAlp F-1  | CCT TTA TGT ATT TTA AAT TTT AG     | esx 5’UTR   | None        |
| esxAUp R-2  | CCT CCT GAA TAT TTT AAG TTC ATC    | esx 5’UTR   | None        |
| esxAIn F-1  | CCA AAG ATG GAC ACA AGC ATT AG     | esx ORF     | None        |
| esxAIn R-2  | CAC CAA TCC AGT TCA TTT CTG       | esx ORF     | None        |
| CapIn F-1   | GCG CTA TG GCA TAT TTC CTG TC     | capA5 ORF   | None        |
| CapIn R-2   | GGT GAA TAC TTA TCA TTC TTT TCC   | capA5 ORF   | None        |
| BioSpo41    | GAG TAT AAT TTT TTA TTT ACA TAT AAA TAA AAA GGC AAT AAT GCG GTT TAA AAG TTA TTA AT | capA5 5’UTR | 5’ Biotin   |
| Spo41-F     | GAG TAT AAT TTT TTA TTT ACA TAT AAA TAA AAA GGC AAT AAT GCG GTT TAA AAG TTA TTA AT | capA5 5’UTR | None        |
| Spo42-R     | ATT AAT TAC TTT TAA ACC GCA TTA TTT TCG CCT TTT TAT TTA TAT GTA AAT TAA AAA TAA TTA ATA TAC TC | capA5 5’UTR | None        |
| SpoMut33-F  | GAG TAG CCG CCT TTT CGC CGG ACA TAT A | capA5 5’UTR | None        |
| SpoMut34-R  | TAT ATG TCC GGC GAA AAG GCG GCT ACT C | capA5 5’UTR | None        |
| Bvls27F     | GGT AAT AGT TTT CCT AAG G           | vlsE ORF    | 5’ Biotin   |
| Bvls77F     | GTA CAG GTT CGT TGG A               | vlsE ORF    | 5’ Biotin   |
| vlsR10      | ACC AAC AGA ACC TGT AC             | vlsE ORF    | None        |
| vlsR4       | CTG GTG TCC CGC GTG GTA G           | vlsE ORF    | None        |

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Electromobility Gel Shift Assays (EMSA)

Recombinant Proteins

Purified B. burgdorferi B31 DNA was used as template to clone the borrelial spoVG gene into pET101, creating pBLJ132. Similarly, the S. aureus and L. monocytogenes spoVG genes were individually cloned into pET101 (Invitrogen, Grand Island, NY), producing pBLJ305 and pBLJ340, respectively. Each cloned insert was completely sequenced to confirm that the spoVG gene was free of mutations and in-frame with the hexa-histidine tag. *Escherichia coli* Rosetta-2 (Novagen, EMB Millipore, Billerica, MA) was independently transformed with pBLJ132, pBLJ340, or pBLJ305. Recombinant proteins were induced by the addition of 1 mM IPTG, and purified using MagnaHis Ni-Particles (Promega, Madison, WI). In order to create conditions conducive to protein-DNA interactions, each spoVG protein was dialyzed against a buffer containing 100 mM dithiothreitol, 30 mM Tris-HCl, 25 mM KCl, 10% glycerol vol/vol, 0.01% Tween-20, 1 mM phenylmethanesulfonyl fluoride [25,26,29,48]. Protein purities and concentrations were assessed via SDS-PAGE and Bradford analyses (Bio-Rad, Hercules, CA) respectively. Protein aliquots were snap frozen in liquid nitrogen and stored at −80°C.

To generate mutant spoVG proteins, site-directed mutagenesis was performed on wild-type plasmid clones, as previously described [49]. Each plasmid was sequenced to ensure that the clones were free of mutations. These constructs were then used as templates for PCR amplification, and cloned into pCR2.1 (Invitrogen, Grand Island, NY, USA), generating pBLJ506, 507, 508, and 509, respectively. Each plasmid was sequenced to ensure that the clones were free of mutations and in-frame with the hexa-histidine tag. All probe and competitor DNA concentrations were determined spectrophotometrically. When appropriate, competitor concentrations and oligonucleotide annealing efficiencies were also confirmed using relative ethidium bromide-stained band intensities following electrophoresis through native 20% polyacrylamide gels separated by agarose gel electrophoresis and purified using Wizard DNA Clean-up Systems (Promega, Madison, WI) before use as EMSA competitors.

All probe and competitor DNA concentrations were determined spectrophotometrically. When appropriate, competitor concentrations and oligonucleotide annealing efficiencies were also confirmed using relative ethidium bromide-stained band intensities following electrophoresis through native 20% polyacrylamide gels (Invitrogen, Grand Island, NY).

EMSA conditions were essentially the same as those described previously [25,26,29,48]. Protein-DNA binding buffer consisted of 50 mM Tris-HCl (pH 7.5), 1 mM dithiothreitol, 130 mM EDTA, 50 ng/nl poly dI-dC, 2µl/ml phosphatase inhibitor (Sigma, St Louis, MO, USA), 30 µl/ml protease inhibitor cocktail 2 (Sigma, St. Louis, MO). For reactions involving cell extracts *B. burgdorferi* B31 ML16, cells were pelleted, resuspended in the above-described buffer, lysed, and cellular debris cleared by centrifugation. All EMSAs were performed at room temperature (approx. 20°C). Probe concentrations were varied as noted in the text. DNA and protein-bound DNA complexes were separated by electrophoresis through native a 10% polyacrylamide TBE gels (Invitrogen), transferred to a nylon membrane (Thermo Scientific, Waltham, MA), and UV cross linked (Stratagene UV Stratalinker 1800, La Jolla, CA). Nucleic acid probes were detected via chemiluminescence (Thermo Scientific) and visualized by autoradiography. Band densitometry was assessed using ImageJ [http://rsbweb.nih.gov/ij] and dissociation constants (Kd) determined as previously described [50].

Bioinformatic Analyses

Promoter motif and structural analyses were performed using MEME (Multiple Em for Motif Elicitation) [http://meme.sdsc.edu/meme/cgi-bin/meme.cgi]. spoVG amino acid sequences were retrieved from GenBank consortium [http://www.ncbi.nlm.nih.gov/sites]. Amino acid sequences were Muscle aligned [http://www.ebi.ac.uk/Tools/msa/muscle/], with gap penalties set at 10, and a minimum of 1000 iterations. Images and analysis were generated using Geneious [http://www.geneious.com].

Species, strain, and GenBank accession numbers used for the analysis shown in Figure 4 were as follows: *Staphylococcus aureus* Newman, NP_645270.1; *Staphylococcus epidermidis* ATCC 12298, NP_765840.1; *Abiotrophia defectiva* ATCC 49176, ZP_04452046.1; *Bacteroides anthracis* G9241, ZP_00240564.1; *Bacillus cereus* ATCC 14579, NP_829950.1; *Bacillus megaterium* WSH-002, YP_005497349.1; *Bacillus subtilis* 168, NP_387930.1; *Bellebiovir communis* HD100, NP_969591.1; *Borelia burgdorferi* B31, NP_212919.1; *Borelia hermsi* DAH, YP_001884203.1; *Calicidello-strep* saccharolyticus DSM 8903, YP_001179173.1; *Clostridium botulinum* ATCC 3502, YP_001256027.1; *Clostridium perfringens* SM10, YP_699747.1; *Corallovolcoccus coronulosus* DSM 2259, YP_005368402.1; *Desulfuracillus baueri* DSM 2075, YP_003806692.1; *Elsinoconium minutum* Pei191, YP_001875395.1; *Haloplasmata contractile* SSD-17B, ZP_08554794.1; *Helicobacter modesticaldum* Ice1, YP_001679836.1; *Typhovibrio polytropus* DSM 2926, YP_003968016.1; *Kiyfipidus toscan DSM 2912, YP_003587990.1; *Leptotrichia goodfellowii* F0264, ZP_06012807.1; *Listeria monocytogenes* EGD-e, NP_463727.1; *Macrococcus caseolyticus* JSC5402, YP_002561317.1; *Mycococcus xanthus* DK 1622, YP_633228.1; *Prevotella dentalis* DSM 3688, EHO3862.1; *Stigmatella aurantiaca* DW4/3-1, YP_00395432.1; *Spirocheta thermophila* DSM 6192, YP_003074257.1; and *Treponema denticola* ATCC 35405, NP_971943.1.

Multimerization State of SpoVG

15% SDS PAGE and 10% native PAGE were used to evaluate denatured and native masses of SpoVG preparations. Following electrophoretic separation polyacrylamide gels were stained with Coomassie Brilliant Blue.

Size-exclusion column chromatography was also employed to assess SpoVG multimerization in solution. A Superdex 200 10/300 column (GE Healthcare, Catalog No. 17-5175-01) was prepared per the manufacturer’s instructions with a mobile phase consisting of 300 mM NaCl, 25 mM Na2HPO4 (pH adjusted to 7.0 with 5.0 M HCl), 1 mM Na3, and 1% glycerol. The mobile
phase was sterile filtered to 0.22 μm. The flow rate was set to 0.10 mL/minute and elution was monitored at A280. The elution of proteins was calibrated using standards of known molecular weight from GE Healthcare LMW and HML Gel Filtration Calibration Kits. (Catalog Nos: 20-4038-41 and 20-4030-42). From the void volume (V0) of the column was determined by injection of 100 μl of 1 mg/mL blue dextran 2000 (2,000 kDa) in elution buffer with 5% glycerol. Protein standards consisting of thyroglobulin (669 kDa), ferritin (440 kDa), adalase (158 kDa), conalbumin (75 kDa), ovalbumin (43 kDa), carbonic anhydrase (29 kDa), ribonuclease A (13.7 kDa), and bovine lung aprotinin (6.5 kDa) were individually prepared in elution buffer with 5% glycerol at 10 mg/mL. These standards were then diluted such that each individual protein had a concentration of 2.0 mg/mL and injected in 100 μl aliquots. The log of the molecular masses of these standards was then graphed against resulting elution volumes (Ve) as Ve/Vo to produce a linear calibration.

experimental protein samples were then run and compared to this calibration curve to estimate molecular mass. Two independent protein preparations were used for each analysis.

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Author Contributions

Conceived and designed the experiments: BLJ TB BS. Performed the experiments: BLJ AC CLR DC MCM TB. Analyzed the data: BLJ AC MCM TB BS. Contributed reagents/materials/analysis tools: BLJ AC MCM TB BS. Wrote the paper: BLJ BS.

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