Structural and Functional Analysis of the C-terminal DNA Binding Domain of the Salmonella typhimurium SPI-2 Response Regulator SsrB

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In bacterial pathogenesis, virulence gene regulation is controlled by two-component regulatory systems. In Escherichia coli, the EnvZ/OmpR two-component system is best understood as regulating expression of outer membrane proteins, but in Salmonella enterica, OmpR activates transcription of the SsrA/B two-component system located on Salmonella pathogenicity island 2 (SPI-2). The response regulator SsrB controls expression of a type III secretory system in which effectors modify the vacuolar membrane and prevent its degradation via the endocytic pathway. Vacuolar modification enables Salmonella to survive and replicate in the macrophage phagosome and disseminate to the liver and spleen to cause systemic infection. The signals that activate EnvZ and SsrA are unknown but are related to the acidic pH encountered in the vacuole. Our previous work established that SsrB binds to regions of DNA that are AT-rich, with poor sequence conservation. Although SsrB is a major virulence regulator in Salmonella, very little is known regarding how it binds DNA and activates transcription. In the present work, we solved the structure of the C-terminal DNA binding domain of SsrB (SsrBc) by NMR and analyzed the effect of amino acid substitutions on function. We identified residues in the DNA recognition helix (Lys179, Met186) and the dimerization interface (Val199, Leu201) that are important for SsrB transcriptional activation and DNA binding. An essential cysteine residue in the N-terminal receiver domain was also identified (Cys45), and the effect of Cys203 on dimerization was evaluated. Our results suggest that although disulfide bond formation is not required for dimerization, dimerization occurs upon DNA binding and is required for subsequent activation of transcription. Disruption of the dimer interface by a C203E substitution reduces SsrB activity. Modification of Cys203 or Cys45 may be an important mode of SsrB inactivation inside the host.

Salmonella infections occur in a wide variety of vertebrate hosts and continue to be a major health problem worldwide for humans. Salmonella infection requires at least two pathogenicity islands, Salmonella pathogenicity islands 1 and 2 (SPI-1 and SPI-2), both of which encode type III secretion systems as well as secreted effectors, chaperones, and regulatory proteins (1–3). Genes located on SPI-1 are required for initial adherence to and invasion of intestinal epithelial cells (4). SPI-2 genes are required for intracellular survival, replication, and systemic infection of Salmonella (5, 6). SPI-2 consists of a 40-kb region located at 31 centisomes on the chromosome and contains ~32 genes (7). SPI-2 genes are organized into functional clusters encoding regulatory, structural genes, effectors, and chaperones. To date 10 promoters of SPI-2 genes and SPI-2 co-regulated genes have been identified (8–10). These promoters, located upstream of the ssrA, srb, sseA, sseB, ssaM, sseI, srfN, sifA, and sifB genes, transcribe genes either individually or in large operons. Transcription from these promoters is activated by the SPI-2 encoded two-component signal transduction system, SsrA/SsrB (8, 11–13).

Two-component signal transduction systems regulate gene expression in response to specific environmental signals (for review see Ref. 14). These systems represent the major paradigm for signal transduction in prokaryotes. They are frequently involved in regulating expression of virulence genes in pathogenic bacteria and are also present in the archaea, lower eukaryotes, and plants. In its simplest form, a two-component system contains a sensor kinase, often a membrane protein that functions in trans-membrane signaling, and a response regulator, usually a DNA-binding protein that regulates transcription. In Salmonella enterica serovar typhimurium, the SsrA/SsrB two-component system controls expression of SPI-2 genes as well as several non-SPI-2-encoded virulence genes (8, 11–13). These genes encode effector proteins that are secreted through the SPI-2 type III secretion system or are structural components of the secretory apparatus (5, 11).

SsrA is a tripartite kinase composed of an ATP binding domain, a histidine phosphorylation domain, a receiver or

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2 The abbreviations used are: SPI, Salmonella pathogenicity island; EMSA, electrophoretic mobility shift assay; r.m.s.d., root mean square deviation; BMM, bis-maleimidodihexane; NOE, nuclear Overhauser effect; NOESY, NOE spectroscopy; HSQC, heteronuclear single quantum correlation; DTT, dithiothreitol.
phosphorylation domain also present in response regulators and a histidyl phosphotransfer domain. SsrA is located in the bacterial inner membrane and, based on homology to similar histidine kinases, has two transmembrane domains. These complex domain structures suggest a phosphorylation whereby the phosphoryl group is transferred via intramolecular reactions from the histidine to an aspartate, to a histidine, and then onto the conserved aspartate of the SsrB response regulator (8, 9, 13, 15).

SsrB is a two-domain response regulator in the NarL/FixJ subfamily. The N-terminal receiver domain of SsrB contains the conserved aspartic acid phosphorylation site and the C-terminal effector domain binds DNA (13). The receiver domains are highly conserved, whereas the effector domains are more tailored to their specialized output functions. NarL, a response regulator involved in nitrogen sensing, is the first structure of this subfamily to be solved and it is also the first structure of a full-length response regulator (16). The structure elucidates many details of the activation mechanism, as the recognition helix is physically blocked by the N terminus. Phosphorylation drives a conformational change that relieves this inhibitory effect, exposing the recognition helix and promoting dimerization (17). SsrB functions by a similar mechanism (13). To activate transcription, SsrB must be phosphorylated at Asp\(^{\text{182}}\) (13), yet surprisingly, overexpression can activate SPI-2 genes in the absence of the SsrA kinase (8, 13). This result indicates that alternative inputs in the form of additional kinases or small molecule phosphodonor molecules such as acetyl phosphate must exist that can phosphorylate SsrB. In this manner the SsrA/SsrB system is uncoupled, as is expression of the srrA/ssrB genes (9, 13).

SsrB is homologous at the primary sequence level to several other two-component regulators for which structures have been determined, including NarL, RcsB, and DosR (16, 18–21). Although this subfamily shares significant structural similarity, important functional differences exist. DosR forms a unique tetramer on the DNA, forming a dimer of dimers, although its relevance in vivo remains to be established (21). NarL binds to its DNA targets as a homodimer, whereas RcsB can bind to DNA either as a homodimer or as a heterodimer with RcsA (22). NarL binds to a well conserved DNA consensus sequence (23), whereas the RcsB binding site is more degenerate (22), and it is difficult to discern a specific binding motif for SsrB\(_c\) (8). DosR binds to a pseudopalindrome that is GC-rich (19). The DNA binding domain of SsrB\(_c\) alone can function as a transcription factor in vivo (13), but the DNA binding domain of NarL cannot, even though it is capable of DNA binding (17). It was therefore of interest to determine how these differences in function might be reflected as structural differences between the subfamily members.

In the present work, we solved the solution structure of SsrB\(_c\) by NMR and examined the effect of amino acid substitution on DNA binding, dimerization, and transcription. The C-terminal 75 amino acid residues of SsrB (SsrB\(_c\)) fold into a four-helix bundle, and the SsrB\(_c\) dimerization surface is similar to that of DosR, NarL, and RcsB. SsrB\(_c\) binds to regions of DNA that are AT-rich with poor sequence conservation. We identified residues in the DNA recognition helix and the dimerization interface that are important for SsrB transcriptional activation and DNA binding. An essential cysteine residue located in the N-terminal receiver domain was also identified, and the effect of Cys\(^{203}\) on dimerization was evaluated. Our results suggest that although disulfide bond formation is not required for dimerization, dimerization occurs upon DNA binding and is required for subsequent transcriptional activation. Disruption of the dimer interface by substitution of Cys\(^{203}\) with a negatively charged residue substantially reduces SsrB activity. Cys\(^{203}\) modification may represent an important mode of SsrB inactivation inside the host.

**EXPERIMENTAL PROCEDURES**

**Bacterial Strains, Plasmids, and Culture Conditions**—A list of the bacterial strains and plasmids used in this study is included in supplemental Table 1. DH5\(\alpha\) was used for routine cloning and BL21(DE3) for protein expression. Bacterial cultures were grown in LB broth or N9 minimal medium (8) as indicated. Antibiotics, where required, were used at the following concentrations: ampicillin 100 \(\mu\)g/ml, chloramphenicol 30 \(\mu\)g/ml, and kanamycin 50 \(\mu\)g/ml.

**Site-directed Mutagenesis**—Site-directed mutagenesis was performed using QuikChange (Stratagene). Plasmids pKF43 (full-length SsrB) and pKF104 (C-terminal domain) were used as templates (13). Oligonucleotide primers ordered from Sigma are listed in supplemental Table 2. Primer pairs C45Sf and C45Sr, K179Af and K179Ar, E182Af and E182Ar, T183Af and T183Ar, M186Af and M186Ar, V197Af and V197Ar, T198Af and T198Ar, L201Af and L201Ar, N202Af and N202Ar, C203Af and C203Ar, C203Ef and C203Er, and C203Sf and C203Sr were used to introduce the C45S, K179A, E182A, T183A, M186A, V197A, T198A, L201A, N202A, C203A, C203E, and C203S substitutions. Substitutions introduced into full-length SsrB in plasmid pKF43 resulted in plasmids pRC32 (C45Sf, pRC24 (K179Af, pRC32 (E182Af, T183Af, M186Af, V197Af, T198Af, L201Af, N202Af, C203Af and C203Ar, C203Ef and C203Er, and C203Sf and C203Sr were used to introduce the C45S, K179A, E182A, T183A, M186A, V197A, T198A, L201A, N202A, C203A, C203E, and C203S substitutions. Substitutions introduced into SsrBc in plasmid pKF104 resulted in plasmids pRC39 (C203A), pRC44 (K179A), pRC45 (M186A), pRC27 (V197A), pRC28 (T198A), pRC29 (L201A), and pRC30 (N202A). Substitutions introduced into SsrBc in plasmid pKF104 resulted in plasmids pRC39 (C203A), pRC44 (K179A), pRC45 (M186A), pRC47 (V197A), pRC48 (T198A), pRC49 (L201A), and pRC50 (N202A). The resulting plasmids were verified by sequencing.

**β-Galactosidase Assays**—Plasmids expressing SsrB and substituted SsrB proteins were transformed into strain MJW604, and β-galactosidase assays were performed as described previously (13). Briefly, cells were grown to \(A_{600} \sim 1.0\), at which time arabinose was added to a final concentration of 0.1%. Following a further incubation of 2 h, samples were taken and β-galactosidase assays performed according to the method of Miller (24). Results shown are the average of four independent cultures.

**Transdominance Analysis**—Plasmids expressing wild-type SsrB and substituted SsrB proteins were transformed into strain MJW704, which contains an intact chromosomal copy of the ssrB gene. Strains were grown overnight in LB and subsequently subcultured into N9 minimal medium. Following 7 h of growth in N9 at 37 °C, arabinose was added (0.1% w/v), and the cultures were incubated at 37 °C for an additional 1 h. At this point samples were taken for β-galactosidase assays, performed as described above.
Expression of SsrBC protein was induced by the addition of arabinose (0.1%) for 3 h at 37 °C. 50 ml of culture was pelleted and stored at −20 °C until required. For protein purification, pellets were resuspended in 3 ml of lysis buffer (50 mM NaH2PO4, 300 mM NaCl, 10 mM imidazole, pH 8.0) containing 100 µg/ml lysozyme and sonicated (Fisher Scientific Sonic Dismembrator, model 100, 3 × 30 s, setting 7 with 2 min on ice between each pulse) to ensure complete lysis and shearing of DNA. Lysates were then incubated with 100 µl of nickel-nitritoltriacetic acid resin for 1 h at room temperature with shaking. The resin was pelleted, washed five times with wash buffer (50 mM NaH2PO4, 300 mM NaCl, 20 mM imidazole, pH 8.0), and eluted in 300 µl of elution buffer (50 mM NaH2PO4, 300 mM NaCl, 250 mM imidazole, pH 8.0). Glycerol was added to a final concentration of 5%, and proteins were stored at −70 °C until required.

**Electrophoretic Mobility Shift Assay**—Electrophoretic mobility shift assays (EMSA) were performed using the Lightshift chemiluminescence EMSA kit (Pierce) according to the manufacturer’s instructions. A 300-bp fragment of the sseI promoter containing the SsrB binding site was amplified using biotinylated oligos B-srflHf and B-srflHr (supplemental Table 2). 20 fmol of biotinylated sseI DNA was used in a 15-µl reaction containing binding buffer (10 mM Tris, pH 7.5, 50 mM KCl) along with 2.5% glycerol, 5 mM MgCl2, 50 ng/µl poly(dl-DC), and 0.05% Nonidet P-40. SsrBC protein at the concentrations indicated was added, and samples were separated by electrophoresis on 5% nondenaturing acrylamide gels run in 0.5 × Tris acetate buffer with EDTA. Following electrophoresis, DNA was electro-transferred to a nylon membrane and detected using the biotin detection system (Pierce).

**BMH Cross-linking**—BMH cross-linking was performed as described previously (25). Approximately 10 µM SsrBC protein was used in the reactions with 50 µM BMH. We also added sseI promoter DNA (1:1 molar ratio) to determine whether the presence of DNA enhanced or interfered with dimer formation. Samples were separated by PAGE on 12% gels and stained with Coomassie Brilliant Blue.

**Protein Expression and Purification for NMR**—SsrBC protein was uniformly labeled with 15N and 13C while being overexpressed in *Escherichia coli* strain BL21(DE3) at 37 °C in M9 minimal media containing [15N]NH4Cl and [13C]glucose. Expression of SsrBC protein was induced by the addition of 0.1% arabinose and grown overnight as described previously (13). The cells were harvested by centrifugation, and the Histagged protein was purified using nickel-nitritoltriacetic acid resin under denaturing conditions according to the manufacturer’s instructions (Qiagen). Purified SsrBc protein was renatured by dialysis against a buffer containing 50 mM Na2HPO4, pH 6.5, 50 mM NaCl, and 5 mM Na2S2O4. The protein concentrations used for NMR analysis ranged from 0.4 to 1 mM as measured by the Bio-Rad protein assay using bovine serum albumin as the standard. Western blots were performed as described previously (13).

**NMR Spectroscopy and Data Processing**—All NMR experiments were performed at 35 °C on a Bruker Avance 800 MHz equipped with a cryoprobe and pulsed-field gradients. The spectra were processed using the software package Triad. The backbone and side-chain resonance assignments were obtained through standard double and triple nuclear experiments with the TOPSPIN software supplied with the instrument. A mixing time of 120 ms was used in all NOESY experiments to generate distance constraints for structure calculations. The assignment of the sequence-specific backbone resonance assignment of 69 amino acid residues of SsrBC was achieved through a combination of standard two-dimensional and three-dimensional double and triple resonance NMR experiments (26). More than 75% of all side-chain resonances were assigned for SsrBC.

**Structure Determination**—The structure determination was based on NOE-derived distances, dihedral angles, and hydrogen bond distance constraints. The NOE distance constraints were obtained from standard three-dimensional 15N- and 13C-edited NOESY-HSQC experiments. The backbone dihedral angle constraints were determined using the program TALOS (27). Hydrogen bond distance constraints were obtained from the hydrogen-deuterium exchange experiments in conjunction with the intermediate range NOE and the secondary structural information. The structures of SsrBC were calculated with the DYANA program (28). Initial structures were calculated using unambiguous NOEs derived from 13C-, 15N-, and 1H-enriched SsrBC. Based on the initial structures, further assignments of the NOE peaks were assigned. A total of 200 structures were calculated using DYANA by the standard simulated annealing protocol and NOE distance constraints, dihedral angles, and hydrogen bond distance constraints. In the final analysis, 20 structures with the lowest DYANA energy and constraints violation energy were selected and examined by PROCHECK-NMR (29). The structures were analyzed using the MOLMOL program (30).

**RESULTS**

**Resonance Assignment and the Structure of SsrBC**—We solved the solution structure of SsrBC by high-resolution 1H, 15N, and 13C NMR spectroscopy. Initial studies with the full-length SsrB protein were complicated because of its extreme insolubility. We had shown previously that the isolated C-terminal domain for the NMR structural studies reported herein.

A 1H-15N HSQC spectrum of 15N-labeled SsrBC with corresponding residue assignments is shown in Fig. 1. In this spectrum, expected correlation peaks for the backbone amide resonances were assigned. The pattern of short and medium range NOEs and 3JHNHα coupling constants served to identify the secondary structural elements of SsrBC (Fig. 2). The pattern of dNN(i, i + 2), dNα(i, i + 3), and dαα(i, i + 4) NOE connectivities with 3JHNHα coupling constants smaller than 6 Hz indicated the presence of four α-helices (Leu150-Ile161, α-1;...
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The acquired at 35 °C and pH 6.5. The resonance assignments are indicated in the backbone heavy atom traces superimposed for residues 15–75 are displayed.

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Gly169–Lys173, A

Figure 1. An assigned HSQC spectrum of SsrB. The spectrum was acquired at 35 °C and pH 6.5. The resonance assignments are indicated in the figure. The horizontal bars connect the side chain NH2 groups to Asn and Glu.

Figure 2. A, an ensemble of the 20 final DYANA structures is shown. The backbone heavy atom traces superimposed for residues 15–75 are displayed. B, ribbon diagram of SsrB. The location of the four α-helices from the SsrB three-dimensional structure are labeled H1–H4. The N and C termini are indicated.

Gly169–Lys173, α-2; Ser177–Met186, α-3; and Val197–Arg205, α-4).

After resonance assignment, the three-dimensional structure was calculated using the program DYANA. A total of 814 interproton restraints and 37 dihedral angle constraints were used in the final round of the structural calculation. For the final structural calculation, the N-terminal 14 residues from the interdomain linker were excluded because of their disordered nature. At the last stage, 100 conformers were calculated, and of these, 20 DYANA conformers with the lowest target function were selected to represent the structure (Fig. 2A). The coordinates have been deposited in the Research Collaboratory for Structural Bioinformatics Protein Data Bank with accession code 2JPC. The r.m.s.d. of the well structured regions of the 20 best structures was 0.62 ± 0.12 Å for backbone atoms and 1.61 ± 0.22 Å for all non-hydrogen atoms. The quality of the structures is high, with no dihedral angles in the disallowed regions. The structural statistics are summarized in Table 1.

The structure of SsrB consists of a compact bundle of four well folded α-helices encompassing residues 143–212 (labeled H1–H4 in the ribbon diagram in Fig. 2B). The three-dimensional structure of SsrB is stabilized by hydrophobic residues, which are conserved among the NarL subfamily members (Fig. 3). The averaged structure from the current NMR study aligns well with the x-ray structure of NarL, with an r.m.s.d. value of 1.62 Å (Fig. 4A) and with DosR of 1.4 Å (Fig. 4B).

SsrB-DNA Interactions and Contact Residues—Thus far, we have identified 10 genes that are directly activated by SsrB; they are AT-rich but do not contain a well conserved consensus site (8, 10, 12, 13). This may be in part because, at many promoters, SsrB acts primarily to counter the effects of gene silencing by H-NS, rather than as a direct transcriptional activator (8, 32). SsrB acts primarily to counter the effects of gene silencing by H-NS, rather than as a direct transcriptional activator (8, 32). DosR binds as a dimer of dimers, forming a unique tetramer and recognizing a conserved DNA binding site (21).

Although the structures of NarL and RcsB are highly homologous, they exhibit very different DNA binding patterns. NarL binds as a homodimer to highly conserved, specific DNA sites exhibiting anti-parallel symmetry (17). RcsB can bind to diverse DNA targets as either a homodimer or as a heterodimer with RcsA (20). DosR binds as a dimer of dimers, forming a unique tetramer and recognizing a conserved DNA binding site (21). An alignment of the recognition helix (H3) of SsrB and its homologues, NarL and RcsB, is shown in Fig. 5A along with other NarL family members. Known or predicted DNA contact

| TABLE 1 |
| --- |
| Data collection and refinement statistics |
| NMR-derived restraints |
| Total interproton restraints | 814 |
| Intra-residue (|j| = 0) | 118 |
| Sequential (|j| = 1) | 249 |
| Medium range (1 < |j| < 5) | 196 |
| Long range (|j| > 5) | 191 |
| Hydrogen bonds | 60 |
| Dihedral angles (ϕ, ψ) | 37 |

Residual violations:
- DYANA target function | 0.34 |
- Upper limit | 2.7 |
- Maximum (Å) | 0.11 |
- Van der Waals | 2.2 |
- Maximum (Å) | 0.12 |

Average r.m.s.d. to mean structure:
- Backbone atoms N, Ca, C (Å) | 0.62 ± 0.12 |
- All heavy atoms (Å) | 1.61 ± 0.22 |

Ramachandran plot (% residues)
- Residues in most favored regions | 73.0 |
- Residues in additional allowed regions | 19.3 |
- Residues in generously allowed regions | 7.7 |
- Residues in disallowed regions | 0.0 |

a Under residual violations, the values are means ± S.D.

b Residues are in secondary elements.
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residues are underlined. In the best studied member of this family, NarL, Lys\(^{185}\) accommodates DNA sequence variation in the three different complexes that have been examined thus far (23). It provides “flexible specificity,” recognizing the major groove floor directly and/or via bridging waters. Val\(^{189}\) is a highly conserved NarL residue that enforces significant DNA base distortion (17, 23, 33). Lys\(^{189}\) of NarL is also important for recognition, by hydrogen bonding to guanines at regions of high DNA helical writh. It is noteworthy that whereas RcsB has only one of these residues (corresponding to Lys192 of NarL), none of them are conserved in SsrB (Fig. 5A). In place of the conserved Val\(^{189}\) of NarL, SsrB has a threonine and RcsB a serine residue. In SsrB, a negatively charged glutamic acid residue replaces Lys\(^{185}\) of NarL, whereas in RcsB, it is replaced by a serine. However, both SsrB and RcsB have a lysine further upstream that corresponds to Lys\(^{179}\) of DosR, a known DNA contact residue (21). Lys\(^{192}\) of NarL, although conserved in RcsB, is replaced with a methionine in SsrB. Altogether, these alignments raise interesting questions with respect to DNA binding specificity in these NarL homologues. RcsB and NarL both share one conserved DNA contact residue, as do RcsB and SsrB. SsrB and NarL share no conserved residues, raising the possibility that SsrB recognizes DNA differently from NarL, although our NMR results suggest that the DNA contact surface of SsrB is similar to that of NarL.

Thus, we assume that the \(\alpha\)-3 helix of SsrB\(_{c}\) is the major groove DNA contact surface, as has been shown in co-crystal structures of the other regulators listed here. Based on the structures of the NarL-DNA, TraR-DNA, and DosR-DNA complexes, we modeled SsrB\(_{c}\) bound to DNA (Fig. 6). The residues predicted to be involved in SsrB-DNA interactions are Lys\(^{179}\), Thr\(^{183}\), and Met\(^{186}\) (highlighted in Fig. 6A).

Glu\(^{182}\) is predicted to make nonspecific contacts with the phosphate backbone. In another view, Val\(^{187}\) and Leu\(^{201}\) may also be DNA contact residues, although they are in the loop and the beginning of the dimerization helix H4 (Fig. 6B).

**Substitution of Predicted DNA Contact Residues Disrupts Transcription**—To identify which of the predicted DNA contact residues are important for SsrB function, we performed site-directed mutagenesis and determined the ability of the mutant proteins to activate transcription. Lys\(^{179}\), Glu\(^{182}\), Thr\(^{183}\), and Met\(^{186}\) were each substituted with alanine, and the ability of the mutant proteins to activate expression of a \(srfH\)-\(lacZ\) fusion strain was determined. Previous studies have shown that sseI is highly dependent on SsrB for transcriptional activation (11, 13), and DNase I footprinting has identified an SsrB binding site between –122 and –95 upstream of the transcriptional start site (13). SsrB and its mutant derivatives were expressed from an arabinose-inducible promoter in strain MJW604, and \(\beta\)-galactosidase assays were performed to determine the transcriptional activation of \(srfH\)-\(lacZ\) (Fig. 7). In the absence of SsrB, essentially no transcription from the \(srfH\) promoter was detected (Fig. 7, column 1), whereas expression of SsrB strongly induced \(srfH\) (Fig. 7, column 2, set to 100%). Transcriptional activation was completely dependent on the phosphorylated Asp\(^{56}\) (Fig. 7, column 3) as shown previously (13). The mutants Lys\(^{179}\)A completely abolished \(srfH\) transcription (Fig. 7, column 4), whereas Met\(^{186}\)A resulted in an ~4-fold decrease in activity compared with the wild type (Fig. 7, column 7). In contrast, substitution of Thr\(^{183}\) with alanine improved the ability of SsrB to activate \(srfH\)-\(lacZ\) expression (Fig. 7, column 6), resulting in 50% stimulation over the activity of the wild type. The Glu\(^{182}\) substitution reduced activity to 60%, consistent with a weak effect from a residue predicted to make nonspecific interactions with the phosphate backbone (Fig. 7, column 5).

These results demonstrate that Lys\(^{179}\) and Met\(^{186}\) are both important for \(srfH\) promoter activation by SsrB, whereas Thr\(^{183}\) is not required. Similar results were obtained with the mutants expressed only as an SsrB\(_{C}\) fragment, except for Met\(^{186}\)A (data not shown; see “Discussion”).

FIGURE 3. An alignment of the primary structure of the DNA binding domains of SsrB and its homologues. References for these homologues are as follows (in parentheses): NarL (17, 23), DosR (21), TraR (18), RcsB (20), FixJ (36) and StyR (35). Amino acids indicated in gray are conserved, and the secondary structure is indicated below the sequence.

FIGURE 4. A, overlay of NarL structure (gray) with SsrBc (blue). B, overlay of DosR (red), and SsrB (green). N = amino terminus, C = carboxyl terminus; helices 1–4 are labeled H1–H4. The r.m.s.d. for Cu for SsrB and DosR is 1.4 Å and for SsrB and NarL is 1.62 Å.

FIGURE 5. Sequence alignment of the DNA contact helix and dimerization helix of SsrB with NarL family members. A, primary sequence alignment of DNA contact helix (α9/HEL) of SsrB with NarL family members for which the structure is known. Amino acid residues of NarL family members known or predicted to be involved in DNA contact are underlined. Predicted DNA contact residues of SsrB are indicated by arrows. B, primary sequence alignment of the dimerization helix of SsrB with NarL, RcsB, and DosR. TraR was not included because its dimerization interface is different (18, 31, 41). Potential amino acid residues involved in dimerization are indicated in red (NarL) and blue (RcsB). A unique cysteine residue is indicated by an asterisk.
Analysis of the SsrBC Dimerization Helix—Sequence alignment of SsrBC helix 4 (H4) with the dimerization helices of DosRC, NarLC, and RcsBC is shown in Fig. 5B. In the co-crystal structure of the NarLC-DNA complex, two hydrophobic residues, Val204 and Val208, reside on the surface of the dimerization helix and engage in intermolecular interactions to form a homodimer on the DNA binding site (17). In the RcsB structure, Ile199 and Asn203 occupy the corresponding positions of the two valines of NarL. RcsB can bind to DNA as a homodimer, but it also binds to DNA as a heterodimer with RcsA (20). The absolute requirement for Val197 and Leu201 indicates that they are important for SsrB activity, and their alignment with dimerization positions in DosR, NarL, and RcsB suggests they are involved in dimerization of SsrB (see “Discussion”). This result also suggests that dimerization is required for SsrB activation of the sseL promoter. Western blots were performed to determine SsrB protein levels in each strain. Comparable levels of SsrB protein were detected with each mutant (see example Fig. 9, inset). In addition, HSQC spectra were acquired on Lys179A and Leu201A mutant proteins, indicating that the proteins are well folded (except for helix 4 of Leu201A; see below).

Role of Cysteine Residues in SsrB Function—Our analysis of the dimerization helix of SsrBC, identified two residues impor-

Our NMR structures indicate that the residues on the surface of H4 are different from the other SsrB family members. To determine which residues in H4 are important for SsrB dimerization, the potential dimerization residues were substituted independently with alanine and the transcriptional activity of the mutant proteins determined in the sseL-lacZ fusion strain (Fig. 8). Alanine substitutions at positions Val197 and Leu201 resulted in mutant SsrB proteins that were unable to activate transcription, e.g., activity was 13 and 6%, respectively (Fig. 8, columns 3 and 5; where wild type = 100%). In a different alignment, it was suggested that Thr198 and Asn202 may correspond to Ile208 and Asn212 of RcsB; thus, these residues were also substituted and their transcriptional activity determined. Substitutions at positions Thr198 or Asn202 had only a moderate effect on SsrB activity, reducing sseL transcription to 72 and 61%, respectively, compared with the wild type (Fig. 8, columns 4 and 6).

4 F. Bernhard (Freie Universitat Berlin), personal communication.
tant for SsrB activity, Val^{197} and Leu^{201}. Another interesting feature of helix 4 is the presence of a cysteine residue located at position 203. This cysteine in SsrB is not conserved in other family members (Fig. 5B). Sequence analysis of SsrB identified two additional cysteines located in the N terminus at positions 45 and 46. This vicinal configuration and distribution of cysteine residues is unique to SsrB response regulator proteins in *Salmonella* serovars *typhimurium* and *typhi*. The presence of a cysteine residue within H4 raised the possibility that it could play a role in SsrB dimerization and function. We therefore investigated the role of all three cysteine residues in SsrB transcriptional activity.

We initially substituted each of the cysteines with alanine and examined the ability of the mutant proteins to bind DNA and to activate transcription. We used alanine substitutions, even though serine is considered a more conservative replacement, because substitution of the lone cysteine in OmpR with serine prevented phosphorylation (34). β-Galactosidase assays were performed using the *ssel-lacZ* fusion with either wild-type SsrB or SsrB with a D56A substitution as positive and negative controls, respectively (Fig. 9). Induction of SsrB expression resulted in activation of the *ssel* promoter, whereas expression of SsrB D56A did not activate *ssel* (Fig. 9, columns 1–3). Substitution of cysteine at position 203 with alanine had no effect on SsrB activity, indicating that disulfide bond formation is not required for activity (Fig. 9, column 7). Similarly, the substitution C46A had a modest effect on SsrB-dependent transcription (Fig. 9, column 6, 34% higher than wild type). In contrast, the C45A mutant was significantly impaired (9% of wild-type activity) in its ability to activate expression of the *ssel-lacZ* fusion (Fig. 9, column 4). A serine substitution at Cys^{45} was also not functional (Fig. 9, column 5). This result demonstrates an important role for Cys^{45} in SsrB function and indicates that neither Cys^{46} nor Cys^{203} is required for SsrB activity (see “Discussion”). To further investigate the role of Cys^{203}, additional substitutions were constructed at this position. Although serine substitution had only a modest effect (74% of wild type), a glutamic acid substitution reduced activity to 34%. In the NMR structure, the side chain of Cys^{203} interacts with Leu^{192} located in the loop between H3 and H4. When Cys^{203} is substituted with an alanine or a serine, this interaction is likely maintained, whereas a residue with a negative charge at this position can destabilize the helix, disrupting the dimer as well as DNA binding (see Fig. 9 and also “Discussion”). To establish that the differences in *ssel* activity among all substituted forms of SsrB was not due to altered expression levels of the mutant proteins, Western blots were performed to determine SsrB protein levels.

5 I. Zhulin (Oak Ridge National Laboratory), personal communication.
in each strain. Comparable levels of SsrB protein were detected with each mutant (a representative blot is shown in Fig. 9, inset).

Transdominance Effect of Cys45.—To further explore the essential role of Cys45 in the N-terminal receiver domain on SsrB activity, we expressed the SsrB mutant in a strain containing the ssrB-lacZ fusion in the presence of the wild-type chromosomal ssrB gene. The experiment was performed under SsrB-inducing conditions so that both forms of the protein were present in the cell. If the mutant form of SsrB can dimerize with the wild-type SsrB but cannot bind to DNA, the resulting “faulty dimer” will not activate transcription of ssr, and β-galactosidase activity will be reduced compared with the wild-type strain not expressing mutant SsrB. Alternatively, expression of a mutant form of SsrB that is unable to dimerize will not result in the formation of “faulty dimers,” and ssr transcription should not be affected. As expected, an increase in ssr-lacZ expression was observed in the wild-type strain compared with the ssrB null (Fig. 10, columns 1 and 2; wild type = 100%). Overexpression of wild-type SsrB in the wild-type background resulted in a further 2.5-fold increase in expression (Fig. 10, column 3), indicating that normally, chromosomal levels of SsrB are limiting. Similarly, expression of the SsrB mutant C45A in the wild-type background also resulted in a >2-fold increase in transcription (Fig. 10, column 4), similar to that seen when overexpressing wild-type SsrB. Yet when C45A was the only form of SsrB present, it was unable to activate ssr transcription (see Fig. 9). This result suggests that a C45A monomer can dimerize with a wild-type SsrB monomer and that the heterodimer exhibits enhanced wild-type activity (Fig. 10, compare columns 3 and 4). Thus, it would appear that the defect in C45A transcription is a defect in its ability to dimerize, not in its ability to bind to DNA (see “Discussion”). In contrast, overexpressed SsrB mutants D56A, K179A, M186A, V197A, and L201A in the wild-type background all exhibited reduced transcriptional activity at the level of the empty vector control (data not shown), suggesting that the mutants form faulty dimers with wild-type SsrB, which significantly reduce its activity.

H4 Substitutions Affect Dimer Formation.—To further investigate the role of amino acids in the dimerization helix, we performed a series of cross-linking experiments with the homobifunctional reagent BMH and purified SsrB fusion proteins. A series of plasmids were constructed expressing fusion proteins from an arabinose-inducible plasmid in strain MJW704, which contains a wild-type chromosomal ssrB gene. Assays were performed under SsrB-inducing conditions to ensure expression of chromosomal SsrB. β-Galactosidase assays were performed to determine the ssr-lacZ activity with both forms of SsrB in the cell. The results are expressed as a percent, where wild-type chromosomal SsrB is 100%.

FIGURE 10. Transdominance assay using chromosomal wild-type SsrB and plasmid-encoded SsrB or SsrB-C45A. Wild-type or mutant SsrB was expressed from an arabinose-inducible plasmid in strain MJW704, which contains a wild-type chromosomal ssrB gene. Assays were performed under SsrB-inducing conditions to determine the ssr-lacZ activity with both forms of SsrB in the cell. The results are expressed as a percent, where wild-type chromosomal SsrB is 100%.
Structural and Functional Analysis of SPI-2 Regulator SsrB

Substitutions in the Recognition Helix Disrupt DNA Binding—BMH cross-linking analysis suggested a role for Leu^{201} in dimerization, thus accounting for the inability of L201A to activate transcription. SsrBC mutants K179A, M186A, and V197A cross-linked similarly to the wild type, suggesting that these amino acids play a different role in SsrB function (Fig. 11). Lys^{179} and Met^{186} are in the DNA recognition helix of SsrB and are predicted to be DNA contact residues. Based on the co-crystal structure of DosR, Val^{197}, despite its position within H4, is also predicted to contact DNA (Fig. 6A). Thus, the inability of these mutant proteins to activate transcription likely resulted from an inability to bind to DNA. To examine the DNA binding properties of the SsrBC mutants, we performed electrophoretic mobility shift assays (Fig. 12). A 300-bp fragment of the ssel promoter containing an SsrB binding site (13) was amplified using primers that were biotinylated on the 5' ends. The result is consistent with the lack of transcriptional activation shown in Figs. 7 and 8. In contrast, the K179A and L201A mutants were completely disrupted in their ability to bind DNA, even at >5 μM SsrBC (Fig. 12, C and F). This result is consistent with the lack of transcriptional activation shown in Figs. 7 and 8. In contrast, M186A exhibited an increased affinity for the ssel promoter compared with wild type (−2–3 fold; Fig. 12D). Although M186A bound ssel DNA with higher affinity, it only activated ssel transcription by 24% compared with wild type (Fig. 7). This result suggests that M186A is capable of binding to DNA but is unable either to effectively counter the effects of H-NS silencing or to form a productive interaction with RNA polymerase (see “Discussion”).

DISCUSSION

SsrB is the major virulence factor in S. enterica, regulating expression of genes within and outside of SPI-2 required for systemic infection. Yet surprisingly little is known about how it recognizes DNA and relieves H-NS silencing to activate transcription. In the present work, we used NMR spectroscopy to determine the structure of the C-terminal DNA binding domain of the response regulator SsrB (SsrBC). Our initial attempts to use full-length protein were thwarted because of its rapid insolubility. As our previous studies had demonstrated that the isolated C terminus of SsrB was stable in solution, bound DNA, and activated transcription of SsrB target genes, we used the isolated C terminus in the structural analysis reported herein.

The three-dimensional structure of SsrBC is highly homologous to both NarL and RcsB. It consists of four α-helices and contains a helix-turn-helix DNA binding domain (helicities 2 and 3) and a dimerization helix (helix 4). Based upon primary sequence alignments of SsrB with its homologues for which structures are known, we identified amino acid residues in the DNA contact helix and dimerization helix that we predicted to be involved in DNA binding and dimerization. We tested these predictions by introducing alanine substitutions at each position and determining the ability of the substituted proteins to activate transcription. Four C-terminal substitutions were found to disrupt SsrB activity. Two substitutions (K179A and M186A) were located within the DNA contact helix, and two (V197A and L201A) were within the loop between H3 and H4 and the dimerization

DNA binding pattern similar to the wild type (Fig. 12B), consistent with its lack of effect on transcription (Fig. 9). Similarly, V197A showed a similar pattern of binding but was shifted to higher protein concentrations (e.g. compare shifted bands at 1.3 μM; Fig. 12, B and F), indicating a more apparent binding defect. In contrast, the K179A and L201A mutants were completely disrupted in their ability to bind DNA, even at >5 μM SsrBC (Fig. 12, C and F). This result is consistent with the lack of transcriptional activation shown in Figs. 7 and 8. In contrast, M186A exhibited an increased affinity for the ssel promoter compared with wild type (−2–3 fold; Fig. 12D). Although M186A bound ssel DNA with higher affinity, it only activated ssel transcription by 24% compared with wild type (Fig. 7). This result suggests that M186A is capable of binding to DNA but is unable either to effectively counter the effects of H-NS silencing or to form a productive interaction with RNA polymerase (see “Discussion”).

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helix. The dimerization and DNA binding abilities of each of the substituted proteins were then examined. SsrBC mutant L201A was unable to dimerize or bind to the ssrI promoter, and its structure was substantially disrupted by the substitution. Clearly this is an important hydrophobic residue, because alanine substitution destabilized H4. K179A could dimerize but was unable to bind DNA, even at high protein concentrations. Neither protein was able to activate expression of the ssrI promoter, indicating that both dimerization and DNA binding are essential for transcription.

The specific contribution of Met186 and Val197 to SsrB function remains unclear. Substitution of either residue resulted in a decrease in ssrI expression; however, both proteins were able to dimerize in vitro and bind DNA in vivo, although V197A exhibited reduced affinity for DNA (Fig. 12). Val197 is the first residue in H4; this position is not conserved among SsrB family members. A positively charged residue (Arg or Lys) at this position in the crystal structures of the DNA complexes is shown to contact DNA and resides in the loop between H3 and H4 (17, 18, 20, 21, 23, 35, 36). Thus, we expect that this loop in SsrB also contacts DNA. The reduced activity of V197A and its lower affinity for DNA suggest that it may undergo a structural rearrangement when SsrB binds to DNA. Interestingly, the substitution M186A increased the affinity of SsrB for DNA (Fig. 12D). It is possible that the observed decrease in transcription could result from the formation of a tight protein-DNA complex at the promoter that inhibits RNA polymerase open complex formation or prevents interaction with RNA polymerase (a “positive control” mutant). A more detailed understanding of the specific mechanism with which SsrB activates transcription is required to distinguish between these possibilities, but our in vitro transcription assay suggests the former.6

SsrB has a dual role as a transcriptional activator and in relieving H-NS silencing (8). Understanding which activity predominates at the different SsrB-regulated promoters is crucial to our understanding of the role of SsrB in Salmonella virulence and will be a major focus of future experiments.

Despite extensive structural similarity, the two closest homologues to SsrB (NarL and RcsB) exhibit very distinct mechanisms of transcriptional activation. NarL activates genes by forming homodimers, which bind to a consensus DNA sequence and activate transcription. In contrast, RcsB can form homodimers or heterodimers with RcsA. RcsB homodimers

6 D. Walthers and L. J. Kenney, unpublished results.
and RcsB-RcsA heterodimers regulate distinct sets of genes, adding an additional layer of complexity to RcsB gene regulation (22, 23). We speculated that if SsrB were to dimerize similarly to NarL, then Val197 and Leu201 would be involved in the dimer interface and that both were important for SsrB activity (Fig. 8). However, NarL is distinct among the family members for which co-crystal structures have been solved by forming a dimer with H4 helices parallel to each other. In contrast, the others form more of an “X” with a more limited dimer interface (Fig. 6A). Thus, although the T198A and N202A substitutions had only minor effects on transcription (Fig. 8), they may still be important dimer contacts. A more radical substitution at this position might produce more dramatic effects, but this result may also indicate that the forces driving dimerization of H4 are weak.

A Role for Reduced DNA Specificity in Pathogenesis—Our analysis of the recognition helix of SsrB identified three residues we predicted to be involved in making direct contacts with the DNA. Two of these, Lys179 and Met186, turned out to be important for ssel activation, and Lys179A was shown to be unable to bind the ssel promoter (Fig. 12C). Unlike NarL and RcsB, no consensus DNA binding sequence has been identified for SsrB. Although Lys179 was shown to be important for SsrB binding to the ssel promoter, a direct role may still exist for Met186 and Thr183 in binding to other SsrB-activated genes, in particular those located within SPI-2. Our recent structural analysis of the global regulator OmpR clarified that OmpR is capable of global regulation precisely because it makes very few base contacts, binding to AT-rich DNA and making phosphate backbone contacts (37). Furthermore, OmpR contacts can vary at different promoters, i.e. DNA contacts are different at the porin genes than at the SPI-2 ssaA gene (37). This property of OmpR enables it to become a regulator of horizontally acquired genes during the course of evolution. Like OmpR, SsrB makes few base contacts, binds to AT-rich DNA, and likely makes numerous contacts with the phosphate backbone. Acquisition of an SsrB binding site has been demonstrated to be an important step in the evolutionary divergence of the human pathogen Salmonella enterica and the reptile pathogen Salmonella bongori (10).

It has recently been shown that H-NS represses horizontally acquired genes, including SPI-2 genes (32, 38). The mechanism by which SsrB overcomes H-NS repression is still unknown. Competition for binding or direct recruitment of RNA polymerase are two possible mechanisms, both of which are employed by SsrB at different promoters. If this is the case, the exact mechanism by which SsrB contacts DNA may vary from promoter to promoter, and Met186 and Thr183 may play more important roles at other promoters.

The Importance of Cysteines—Our investigation of SsrB structure and function also led us to examine the role of cysteine residues in SsrB activity. SsrB contains an unusual distribution of cysteine residues for a response regulator. One cysteine is located in the C terminus, within the dimerization helix, whereas two vicinal cysteines are located in the N-terminal receiver domain. Cys45 was shown to be required for function (Fig. 9), although its role in transcription is not yet clear. One interpretation is that C45A substitution causes an allosteric effect that reduces DNA binding in the C terminus. There are many examples in the case of OmpR where phosphorylation site substitutions alter DNA binding and vice versa (33, 34, 39). However, this cannot be the case with Cys45, because in the trans-dominance assay, there was an increase in transcription in the presence of wild-type SsrB, indicating that it is capable of DNA binding (Fig. 10). Cys45 might be involved in dimerization, perhaps by remodeling the dimer interface from an inactive form involving the N terminus to an active form involving H4-H4 interactions in the C terminus. Interestingly, Cys65 is located in α-helix 2 in the receiver domain, not in the typical α-4/β-5/α-5 dimer interface of many response regulators. Its role in SsrB function remains to be elucidated. Although substitution of Cys203 with alanine did not affect transcription (Fig. 9), our NMR results indicate that the Cys203 side chain interacts with Leu192. This interaction is likely maintained when Cys203 is substituted with alanine or serine, but the presence of a negatively charged glutamic acid residue at this position would reduce this interaction, destabilizing H4 and reducing SsrB dimerization, DNA binding, and transcription. Modification of Cys203 in vivo by NO when Salmonella is within the macrophage vacuole may have a similar effect and disrupt SsrB function. This could account for the decreased ability of Salmonella to survive inside activated macrophages (40).

Disruption of SsrB function in this manner is likely to occur via destabilization of H4 and the loop between H3 and H4.

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