Identification of amino acid residues of *Salmonella* SlyA that are critical for transcriptional regulation

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The type III secretion system encoded by *Salmonella* pathogenicity island 2 (SPI-2) is essential for the intracellular survival and replication of *Salmonella enterica*. The expression of SPI-2 genes is dependent on a two-component regulatory system, SerA (SpiR)/SsrB, encoded in the SPI-2 region. This paper shows that SlyA regulates transcription of the sensor kinase SsrA by binding to the ssrA promoter, indicating that SlyA is directly involved in the regulation of SPI-2 gene expression. A structure model of the SlyA dimer in complex with DNA was constructed. The model of SlyA indicated that its structure is very similar to that of other MarR family proteins. Based on this model, site-directed mutagenesis of residues located in the winged-helix region required for DNA binding and in the a-helices of the N-terminal and C-terminal regions required for dimerization of the SlyA protein was performed to identify the residues that are critical for SlyA function. Nine mutants of SlyA with single substitutions were unable to activate ssrA transcription in vivo. These mutant SlyA proteins revealed that the residues Leu-63, Val-64, Arg-65, Leu-67, Leu-70, Arg-86 and Lys-88 within the winged-helix region are required for DNA binding, and residues Leu-12 and Leu-126 within the a-helices of the N-terminal and C-terminal regions are required for efficient dimer formation. A *Salmonella* slyA mutant strain carrying a plasmid expressing SlyA derivatives containing mutations at these amino acid positions did not exhibit restored SlyA function in infected HeLa cells, thereby confirming the structural and functional relationships of the SlyA protein.

**INTRODUCTION**

*Salmonella enterica*, a facultative intracellular pathogen, can replicate within macrophages. The intracellular survival of *S. enterica* serovar Typhimurium requires a number of virulence-associated proteins encoded in *Salmonella* pathogenicity island 2 (SPI-2) on the bacterial chromosome (Cirillo et al., 1998; Hensel, 2000; Ochman et al., 1996; Shea et al., 1996). SPI-2 encodes a two-component regulatory system, structural components of a type III secretion system (TTSS), effector proteins and chaperones, which mediate the secretion of the effectors (Cirillo et al., 1996; Hensel, 2000; Ochman et al., 1996; Shea et al., 1996). SPI-2 TTSS functions to export effector proteins across the SCV membrane and into the cytosol of host cells, resulting in the prevention of the recruitment of NADPH oxidase to the SCV (Gallos et al., 2001; Vazquez-Torres et al., 2000), as well as leading to the rearrangement of cellular microfilaments (Meresse et al., 2001; Miao et al., 2002) and microtubule networks (Brumell et al., 2002; Guignot et al., 2004). Remodelling of the host cell cytoskeleton is thought to be involved in maintaining the integrity of the SCV membrane and directing SCV traffic. In *Salmonella*-infected epithelial cells, the SCV forms long filamentous structures referred to as *Salmonella*-induced filaments (Sifs), which are elongated tubules that appear to extend from the SCV membrane along the host cell microtubules (Guignot et al., 2004).

The expression of SPI-2 TTSS structural components and the associated secreted effectors is induced specifically inside host cells (Cirillo et al., 1998; Shea et al., 1996; Valdivia &...
Falkow, 1997), as well as under in vitro conditions that mimic the intracellular environment of the SCV, which include low osmolarity (Garmendia et al., 2003; Lee et al., 2000), calcium limitation (Garmendia et al., 2003), phosphate limitation (Deiwick et al., 1999) and iron limitation (Zaharakis et al., 2002). The induction of SPI-2 genes in response to these stimuli is dependent on SsrA (also referred to as SpoR)/SrbB, a two-component regulatory system encoded within the SPI-2 region. SsrA is the putative cognate sensor kinase for SrbB, and SrbB is the response regulator (Ochman et al., 1996; Worley et al., 2000). Once activated, SsrB induces the expression of genes located both inside and outside SPI-2 by the direct binding of target gene promoters (Feng et al., 2003, 2004; Garmendia et al., 2003; Miao et al., 2002). Upstream signals that activate SPI-2 gene expression are dependent on two other two-component regulatory systems, OmpR/EnvZ (Feng et al., 2003, 2004; Lee et al., 2000; Worley et al., 2000) and PhoP/PhoQ (Bijlsma & Groisman, 2005; Deiwick et al., 1999). OmpR binds to both ssaR and sscB promoters in vitro (Feng et al., 2003, 2004; Lee et al., 2000), and PhoP is directly involved in sscB transcription and the protein levels of SsrA post-transcriptionally (Bijlsma & Groisman, 2005). In addition to these regulatory systems, it has been reported that SlyA, a virulence-associated transcriptional regulator, controls the expression of ssaRAB in vivo (Feng et al., 2004; Linehan et al., 2005; Navarre et al., 2005). However, it has not yet been determined whether SlyA controls the ssaRAB transcript as a direct or an indirect effect.

The Salmonella slyA gene is implicated in virulence, survival in mouse macrophages, resistance to oxidative stress and resistance to antimicrobial peptides (Buchmeier et al., 1997; Daniels et al., 1996; Kaneko et al., 2002; Libby et al., 1994; Navarre et al., 2005; Shi et al., 2004; Watson et al., 1999). SlyA regulates the expression of a large number of genes during the stationary phase and also in the intracellular environment of host cells (Buchmeier et al., 1997; Daniels et al., 1996). Recently, transcriptome and proteome analyses have identified a large number of SlyA-dependent (activated and repressed) genes belonging to the SlyA regulon (Navarre et al., 2005; Spory et al., 2002; Stapleton et al., 2002). Interestingly, several of these SlyA-activated genes were also controlled by the PhoP/PhoQ two-component regulatory system, including those genes required for virulence and resistance to antimicrobial peptides (Navarre et al., 2005). In addition, slyA itself is activated under low-Mg{\textsuperscript{2+}} conditions by the PhoP protein, which binds directly to the slyA promoter region (Norte et al., 2003; Shi et al., 2004).

SlyA is known as a member of the MarR/SlyA family of transcription regulators, more than 130 of which have been identified thus far in bacteria and archaea (Thomson et al., 1997; Wu et al., 2003). Within this family, SlyA is closely related to RovA from Yersinia, Rap from Serratia marcescens and Hor from Erwinia carotovora. In addition, it is more distantly related to MarR, EmrR, HpaR and HpcR from Escherichia coli; MexR from Pseudomonas aeruginosa; PecS from Erwinia chrysanthemi; SlyA-Ef from Enterococcus faecalis; and Hpr and ScoC from Bacillus subtilis. Moreover, it has been shown to be related to several other regulatory proteins. These regulatory proteins control the expression of resistance to multiple antibiotics, organic solvents, agents that induce oxidative stress, and virulence factors (Alekshun & Levy, 1997). This diverse group of MarR homologues is very similar with respect to protein structure; the protein forms a dimer and contains a winged-helix DNA-binding motif (Alekshun et al., 2001; De Silva et al., 2005; Hong et al., 2005; Lim et al., 2002; Liu et al., 2001; Wu et al., 2003). In these regulatory proteins, the helix–turn–helix motif is followed by a wing composed of two antiparallel β-strands. Therefore, MarR/SlyA family proteins consist of two functional domains, one for protein dimerization and one for DNA interaction.

It has been demonstrated previously that the dimer form of SlyA interacts with the DNA and recognizes a palindromic DNA structure (Stapleton et al., 2002). The 12 bp consensus sequence, TTAGCAAGCTAA, is located in the slyA promoter, and has been identified as the SlyA-binding site, which is involved in the autoregulation of the slyA gene (Stapleton et al., 2002). In several SlyA-regulated genes, DNA sequences (TTAGTTTTTGTCTTAA, located in the uglR promoter; TTTGAATGTTAA, located in the pagC promoter; and TTAGCTGAATA, located in the mig-14 promoter) have been identified as SlyA-specific binding sites by DNase I protection assay (Navarre et al., 2005; Shi et al., 2004). Thus, it is likely that the SlyA homodimer binds preferentially at the inverted repeat with a core TTAG motif.

More recently, it was demonstrated that the inability of a Salmonella slyA mutant strain to survive within macrophages was partly due to the reduced expression of SPI-2 genes (Linehan et al., 2005). Although the mechanism by which SlyA controls ssaRAB transcription remains unknown, it has been suggested that the SlyA protein promotes SPI-2 function via the SsrA/SrbB two-component regulatory system (Feng et al., 2004; Linehan et al., 2005; Navarre et al., 2005). Here, we report that the transcription of the ssa gene and the expression of SPI-2 function in vivo both require the slyA regulatory gene. We show that the SlyA protein binds directly to the ssaR promoter. In order to more fully understand the molecular mechanism involved in ssa gene expression in Salmonella, we constructed a structural model of the SlyA dimer in the complex with DNA. Using the predicted structure of the SlyA–DNA complex, we identified several specific residues in SlyA that are critical for DNA binding activity and for dimer formation. The importance of these residues in the functional domains of SlyA was confirmed by site-directed mutagenesis. In vivo and in vitro analyses of the point-mutated SlyA proteins revealed that the amino acid residues of SlyA involved in the interaction with DNA and in dimerization are critical for the transcriptional activation of the ssa gene.

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**METHODS**

**Bacterial strains, plasmids and growth conditions.** The bacterial strains and plasmids used in this study are listed in Table 1. *E. coli* and *Salmonella* strains were grown in Luria–Bertani (LB) broth or on LB agar under conditions for selection for resistance to ampicillin (100 μg ml⁻¹), kanamycin (25 μg ml⁻¹) and nalidixic acid (50 μg ml⁻¹), as appropriate.

**Plasmid construction and site-directed mutagenesis.** The *slyA* gene of *S. enterica* serovar Typhimurium strain SH100 was cloned into the vector pCACTUS as described previously by insertion mutagenesis and allele exchange using the temperature-sensitive suicide vector pCACTUS as described previously (Miki et al., 1999). A DNA fragment containing the *slyA* gene was amplified by PCR from the genomic DNA of *S. enterica* serovar Typhimurium strain SH100 using primers slyA-N1 (5′-GGCTCGAGGATACGCCACTAGGTTCCTG-3′) and slyA-N2 (5′-GGCTCGAGATCGTAGAGTGCAAATTTCAA-3′). The 445 bp PCR product was digested with *Xho*I and *Pst*I and ligated into the same site of the expression vector, pBAD-HisA (Invitrogen), thereby generating pBAD-slyA. The *slyA* point mutants were constructed by site-directed mutagenesis using pBAD-slyA as a template and the respective oligonucleotide primers, and by using the GeneTailer site-directed mutagenesis system (Invitrogen) according to the manufacturer’s instructions. The constructed plasmids were transformed into *E. coli* DH5α-T1B competent cells (Invitrogen), and the presence of the respective mutation was confirmed by DNA sequencing.

For the complementation assays, the plasmid pMW-slyA was constructed by inserting the *Xho*I–*Pst*I III fragment containing the *slyA* gene from pBAD-slyA into the *Sal*–*Hind*III sites of pMW119 (Nippon Gene).

**Mutant construction.** A nonpolar mutant of *slyA* was constructed by insertion mutagenesis and allele exchange using the temperature- and sucrose-sensitive suicide vector pCACTUS as described previously (Miki et al., 2004). A disruption of the *slyA* gene was created by insertion of the *Sma*I-digested Km*-encoding gene (*kan*) cassette from plasmid pUC18K (Menard et al., 1993), which does not affect transcription of downstream genes, into the *Sal*I site in the coding region of *slyA*. The disrupted genes were then subcloned using *Sal*I and *Sph*I into the similarly digested pCACTUS, and the resulting plasmid was introduced into strain SH100 by electroporation for allele-exchange mutagenesis, thereby generating *slyA* insertion mutant strain SH110 (*slyA::kan*). Chromosomal mutations were verified by PCR analysis.

*Salmonella* strains SH112 and SH113 were constructed by P22 phage transduction of an ssaV::cat mutation from a derivative of strain ATCC 14028s (Gotoh et al., 2003) and a ΔssaV::cat mutation from strain TM114, a derivative of strain SL1344 (Miki et al., 2004), respectively, to the wild-type strain SH100.

To construct a transcriptional fusion of the ssaV promoter region to the promoterless lacZ gene in the integrational plasmid pLD-lacZ (Miki et al., 2004), the DNA fragment containing the ssaV promoter region was amplified by PCR using primers ssaV-Pro (5′-GGGGATCCGAACCCGGCTCGCTCACAACC-3′) and ssaV-RV (5′-GGGGATCCCGCTCGCTAGTGAATTCGCTCACAACC-3′). The PCR product digested with *Sal*I and *Bam*HI was ligated into the same sites of pLD-lacZ, thus producing pLD-ssaV. The resulting plasmids were transferred from *E. coli* SM10::pir to strain SH100 by conjugation, generating strain SH112.

**Expression and purification of the SlyA protein.** For the expression of recombinant SlyA protein, plasmid pBAD-slyA was introduced into *E. coli* strain LMG194 (Invitrogen) and was expressed according to the manufacturer’s instructions. N-terminal His₆-tagged SlyA (His₆-SlyA) fusion protein was purified by affinity chromatography on Ni-NTA agarose (Qiagen).

**SDS-PAGE and Western blot analysis.** The protein samples were separated by SDS-PAGE and transferred to PVDF membranes (Immobilon, Millipore) for immunoblotting. Western blot analysis was carried out as described previously (Miki et al., 2004). The blots were incubated with anti-Penta.His antibody (1 : 1000, Qiagen), and anti-mouse secondary antibody conjugated to alkaline phosphatase (Sigma) was used at a 1 : 10 000 dilution.

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DNA-binding assays. For the binding assays of the immobilized SlyA protein to purified DNA, His₆-SlyA protein was immobilized on Dynabeads M-450 Goat anti-Mouse IgG (Dynal) by anti-Penta.His antibody as described previously (Tobe et al., 1996). Defined PCR fragments carrying different portions of the ssrA promoter region were incubated with Dynabeads-SlyA in 50 μl binding buffer containing 10 mM Tris/HCl (pH 7.9), 5 % (w/v) glycerol, 1 mM EDTA, 1 mM DTT and 400 mM NaCl at 4 °C for 60 min. After the Dynabeads were captured on Dynal MCP (Dynal), the supernatant containing unbound DNA was transferred to new tubes. The Dynabeads were then washed three times in binding buffer. Bound DNA was released from the Dynabeads-SlyA complex by incubation in high-salt buffer containing 10 mM Tris/HCl (pH 7.9), 5 % glycerol, 1 mM EDTA, 1 mM DTT and 2 M NaCl. After precipitation by the addition of ethanol, the DNAs were resuspended in 10 μl TE buffer, and then the samples were analysed by 1.5 % agarose gel electrophoresis. The PCR fragments used for the assays were amplified with the following primers: ssrA-P1 (5'-CATCCGGATTTTCCGATTTACATCGCCA-3'), ssrA-P2 (5'-ACGATTTTATCACTGCATCT-3'), ssrA-A1 (5'-AATTGTCGTAATCTCAAGATTGCCG-3') and ssrA-R1 (5'-GTGTTGCTTAGTACAATTATCATC-3'). The PCR products were purified with a MinElute PCR Purification kit (Qiagen).

For the DNA retardation assays, the binding of SlyA to PCR fragments was carried out in a 20 μl reaction mixture containing purified SlyA protein and 1 μg of each DNA. The reaction buffer contained 10 mM Tris/HCl (pH 7.5), 1 mM EDTA, 50 mM NaCl, 50 mM MgCl₂, 5 mM DTT and 5 % glycerol. The reaction mixtures were incubated for 15 min at room temperature and were subsequently loaded onto 4 % polyacrylamide gels, which were run in 0.5 % TBE and stained with ethidium bromide.

Model of SlyA structure. The model structure of the SlyA–DNA complex was constructed based on homology modelling methods. Searches for reference proteins and sequence alignments were performed using SKE-Chimer (Takeda-Shitaka et al., 2005), which is a web user-interface system for protein structure predictions, through which the selection of reference proteins and sequence alignments are carried out by the analysis of abundant data generated by eight sequence search methods such as PSI-BLAST (Altschul et al., 1997). To assess the quality of homology modelling, the Critical Assessment of Techniques for Protein Structure Prediction (CASP6; http://predictioncenter.org/casp6/meeting/presentations/casp6_Program.doc). Based on the alignment given by SKE-Chimer, FAMS Ligand&Complex (FAMS that constructs protein–ligand complex models based on the structure of the reference protein–ligand complex) successfully constructed a three-dimensional model structure (Ogata & Umeyama, 2000; Takeda-Shitaka et al., 2004a, b, 2005, 2006). After the modelling was complete, the quality of the stereochemistry of the SlyA model was verified. In the model structure, no unfavourable contacts between the atoms and no unnatural chiral centres were observed, and there were no steric hindrances that prevented the close interaction of the two monomers in the homodimer. In the Ramachandran plot of the main-chain φ-ψ angles rendered by the program PROCHECK (Laskowski et al., 1993), almost all of the non-glycine residues were in the most favoured or allowed regions. Moreover, all of the ω angles were trans-planar. In this model structure, DNA from the Corynebacterium diphtheriae DtxR (ClI02D mutant)–DNA complex [Protein Database (PDB, Berman et al., 2000) ID code: 1F51] was included in order to predict the SlyA–DNA interactions.

β-Galactosidase assay. For β-galactosidase assay, S. enterica serovar Typhimurium strain SH122 with the plasmids pBAD-slyA and its derivatives expressing point mutations in SlyA were grown overnight at 37 °C in LB medium with aerobation and diluted at 1:50 into fresh LB broth and grown for 2 h under the same conditions. To induce the expression of the SlyA protein, arabinose was then added at a final concentration of 0.01 % to each of the cultures, which were incubated for an additional 3 h. The activity of the ssrA promoter was estimated by measuring the β-galactosidase activity according to standard procedures with the substrate o-nitrophenyl β-D-galactoside (Miller, 1992). Samples were taken from each culture for Western blot analysis of His₆-SlyA derivatives at the same time the enzyme assay was performed.

DSS cross-linking. Purified samples of His₆-SlyA and its derivatives (15 μg) were incubated with 100 μM disuccimidyl suberate (DSS, Pierce) in a 100 μl reaction mixture containing conjugation buffer (50 mM HEPES, pH 7.5, 200 mM NaCl) at room temperature for 1 h. The reaction was quenched by the addition of 1 M Tris/HCl, pH 8.0, to a final concentration of 25 mM, for 30 min. The cross-linked samples (10 μl each) were analysed on 15 % SDS-PAGE gels and stained with Coomassie brilliant blue.

Cell cultures. HeLa cells were grown in minimal essential medium (MEM, Sigma) supplemented with 10 % fetal bovine serum in the presence of gentamicin (100 μg ml⁻¹) and kanamycin (60 μg ml⁻¹) and were maintained in a humidified atmosphere containing 5 % CO₂ at 37 °C.

Bacterial infection of cultured cells. HeLa cells were infected with exponential-phase Salmonella strains as described previously (Miki et al., 2004). Cells were washed after 15 min exposure to Salmonella and subsequently incubated in medium containing gentamicin to kill extracellular bacteria. For SPI-2 phenotypic analysis of Salmonella-infected HeLa cells, the cells were fixed in 4 % paraformaldehyde in PBS for 30 min at 4 °C. After being washed three times in PBS, the fixed cells were permeabilized in 0.1 % Triton X-100 in PBS for 5 min. For triple immunofluorescence staining, the samples were probed with the following; anti-LAMP-1 monoclonal antibody (H4A3, 1:1000, BD Pharmingen) and Alexa Fluor 488-conjugated goat anti-mouse IgG antibody (1:500, Molecular Probes) for the detection of LAMP-1; anti-Salmonella LPS antiserum (1:1000, Denka Seiken) and Alexa Fluor 647-conjugated goat anti-rabbit IgG antibody (1:500, Molecular Probes) for the detection of Salmonella; and Texas red-X phalloidin (1:500, Molecular Probes) for the detection of F-actin. The samples were then mounted onto slides using Vectashield solution (Vector Laboratories), and were viewed at ×63 magnification on a confocal laser scanning microscope (LSM510 META, Zeiss). Infected HeLa cells were then scored for the presence of Sifs and association of F-actin. The percentages of each strain that were positive for Sifs and F-actin were recorded for three independent experiments in which a total of 150 infected cells were examined for each strain. Values are given as the means ± SD of triplicates. Significance was tested by applying Student’s t test. Error bars in figures represent SD.

RESULTS

SlyA interacts directly with the ssrA promoter region

It has been reported that the dimeric form of SlyA protein interacts with DNA and recognizes short palindromic sequences (Stapleton et al., 2002). In fact, we found that the recognized sequences consist of the palindromes TTATTTTTAAATTAA (designated IR in Fig. 1a), located

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at positions −33 to −20 on the ssrA promoter region, which is similar to the SlyA-binding sequence TTAGCAAGCTAA described for the slyA promoter (Stapleton et al., 2002). Therefore, we prepared a purified His₆-SlyA fusion protein and three different DNA fragments containing segments of the ssrA promoter region (fragment a, 466 bp, from −100 to +368 and fragment b, 356 bp, from −12 to +368) and only ssrA ORF (fragment c, 200 bp, from +168 to +368) (see Fig. 1b) by PCR amplification using the S. enterica serovar Typhimurium SH100 chromosome, and examined the ability of the fragments to bind to the SlyA protein. The Dynabeads–SlyA complex, which immobilized the His₆-SlyA fusion protein on Dynabeads via anti-Penta.His antibody (see Methods for details), was used for the DNA-binding assay. Three different DNA fragments (a, b and c) were incubated with Dynabeads–SlyA, and the bound DNA and the unbound DNA were separated by agarose gel electrophoresis. The binding assays revealed that DNA fragment a was able to bind to Dynabeads–SlyA, whereas fragments b and c were not (Fig. 1c). The same results were obtained when the DNA-binding capacity of purified His₆-SlyA fusion protein for the ssrA promoter region was examined by gel mobility shift assays. Mixtures of DNA fragments encompassing different portions of the ssrA promoter region (a, b and c) were incubated with increasing concentrations of His₆-SlyA fusion protein, and the protein–DNA complex was separated by 4 % PAGE. A single SlyA–DNA complex was found with DNA fragment a, but no complex formation was detected with either fragment b or fragment c (Fig. 1d). These results suggest that SlyA binds to the sequence spanning from nucleotides −100 to −13 of the ssrA promoter.

**Construction of a structural model of the SlyA protein**

SlyA consists of a dimer with two functional domains required for DNA binding and dimerization (Stapleton et al.,...
To obtain structural and functional information about the SlyA molecule, we constructed a SlyA homodimer model using SKE-CHIMERA (Takeda-Shitaka et al., 2005) and FAMS Ligand&Complex (Ogata & Uemeyama, 2000; Takeda-Shitaka et al., 2004a, b, 2005, 2006). The accuracy of comparative models generally depends on sequence identity between the target and the modelled proteins, because errors in alignment or template selection increase for low sequence identities (Baker & Sali, 2001). Thus, to improve model accuracy, we constructed and examined a number of alignments derived from eight sequence search methods using SKE-CHIMERA. Among the candidate reference proteins for SlyA, the X-ray structures of homodimers of E. faecalis SlyA-like transcriptional factor, SlyA-Ef (PDB ID: 1LJ9), P. aeruginosa MexR (PDB ID: 1LNW), B. subtilis YusO protein (PDB ID: 1S3J) and E. coli MarR (PDB ID: 1JGS) were selected, as the sequence identity between SlyA and these four candidates (23 %, 20 %, 19 % and 22 %, respectively) was higher than that of other candidate proteins. Although the sequence identities of these four candidates were not high (less than 30 %), the E-values from sequence searches of these proteins were low enough that these proteins could be used as the reference proteins for SlyA. In addition, the secondary structure prediction using PSIPRED (Jones, 1999) indicated that SlyA had a structure similar to that of these proteins. In order to construct the SlyA–DNA complex model, C. diphtheriae DtxR (C102D mutant), of which the X-ray homodimer structure was complexed with DNA (PDB ID: 1F5T), was selected as another candidate reference protein. In 1F5T, z-helix C in each monomer bound in the major groove of the DNA. The structural comparison between 1F5T and the former four candidates (1LJ9, 1LNW, 1S3J and 1JGS) showed that the spacing between the z-helix C of one monomer and the z-helix C’ of the other monomer in 1F5T was similar to that between the z-helix z4 of one monomer and the z-helix z4’ of the other monomer in 1S3J. Therefore, we superimposed 1F5T onto 1S3J by fitting z-helix C and z-helix C’ onto z-helix z4 and z-helix z4’, respectively, and we constructed a model of the complex structure of B. subtilis YusO protein from 1S3J and DNA from 1F5T. The present model of the SlyA–DNA complex was predicted by FAMS Ligand&Complex based on the alignment shown in Fig. 2(a) using the structure of this YusO and DNA complex as a reference.

Identification of amino acid residues of SlyA that are critical for DNA binding

As shown in Fig. 2(b), the winged-helix motifs are positioned to interact with a palindromic recognition sequence, with specific contacts expected to occur predominantly between z-helix z4 and the wing, and the major groove of the DNA. Therefore, we mutated the slyA gene in plasmid pBAD-slyA at 16 positions in z4 (Pro-61, Ser-62, Leu-63, Val-64, Arg-65, Thr-66, Leu-67, Asp-68, Gln-69, Leu-70, Gln-71 and Asp-72) and in the wing (Arg-85, Arg-86, Lys-88 and Arg-89), in order to replace each of these residues with an alanine. Plasmids harbouring the mutated slyA gene were transformed into the slyA null mutant strain. Considering that mutations that lead to an instability of the SlyA protein could lead to a decrease in its in vivo activity, we tested the production of each mutant SlyA protein by immunoblot analysis using an anti-Penta.His monoclonal antibody. The slyA null mutant strain harbouring an empty plasmid vector pBAD-HisA was used as a negative control. All mutant proteins were expressed at levels comparable to that of the wild-type SlyA under the conditions used in this study (Fig. 3a). To investigate the importance of these residues for DNA binding, the ability of these mutants to promote the expression of srrA-lacZ reporter gene fusion was examined. Six of the 16 mutant SlyA proteins, i.e. L63A, R65A, L67A, L70A, R86A and K88A, were completely unable to activate srrA-lacZ expression (Fig. 3b). In addition, the loss of the DNA-binding capacity of these purified mutant SlyA proteins in the srrA promoter region (fragment a in Fig. 1c) was confirmed by gel mobility shift assays (Fig. 3c). Similarly, these six SlyA mutant proteins were unable to bind to the DNA fragment containing the slyA promoter region (nucleotides −103 to +51) (data not shown). These results strongly suggest that six residues in the z-helix z4 and wing region are critical for the DNA-binding activity of SlyA. Furthermore, it was of note that the purified mutant SlyA protein V64A showed the ability to bind to all of the DNA fragments tested. In addition, V64A appeared to form higher-molecular-mass DNA complexes (data not shown), thus indicating that a mutation in Val-64 also generates a nonfunctional protein. A sequence alignment of the DNA-binding domain of SlyA revealed that these residues are highly conserved in proteins more closely related to SlyA, including RovA, Hor and Rap, but that they are not conserved in proteins more closely related to MarR (Fig. 4).

Identification of amino acid residues of SlyA that are important for dimerization

To identify the amino acid residues that are required for the dimerization of SlyA, we searched protein–protein interaction sites as described previously (Terashi et al., 2005), and then individually mutated residues that are thought to be involved in protein–protein interactions within z-helices z1 (Leu-9, Leu-12, Leu-15 and Leu-19) and z6 (Ile-114, Ile-118, Leu-126, Leu-129, Ile-130 and Leu-133) to alanine. Plasmids harbouring the mutated slyA gene were introduced into the Salmonella slyA null mutant strain, and the levels of expression of these point mutants of SlyA were assayed to determine their abundance in vivo by immunoblot analysis using an anti-Penta.His monoclonal antibody. All of the alanine substitutions showed levels of expression similar to that of the wild-type (Fig. 5a). Each SlyA mutant protein was then tested for its ability to activate the srrA promoter in the Salmonella slyA mutant strain harbouring the srrA-lacZ fusion gene. Two of 10 mutant SlyA proteins (L12A and L126A) were significantly defective in terms of the activation of srrA transcription (Fig. 5b). In order to examine the effects of Leu-12 and Leu-126 mutation on the dimerization of SlyA, the L12A and L126A mutant SlyA proteins were
Fig. 2. Homology modelling of the SlyA–DNA complex. (a) Sequence alignment between SlyA and its reference structure of B. subtilis YusO (PDB ID: 1S3J). The numbering of the SlyA primary sequence is indicated above the sequences. The secondary structure elements are indicated under the sequences. X in YusO (1S3J) indicates selenomethionine. The regions of SlyA that were predicted to be α-helices and β-strands by PSIPRED (Jones, 1999) are highlighted in red and blue, respectively. The regions of YusO (1S3J) that were assigned as α-helices and β-strands by STRIDE (Frishman & Argos, 1995) are highlighted in red and blue, respectively. (b) Stereoview of the homology model of the SlyA–DNA complex. The two molecules of SlyA are shown in orange and green. The secondary structure elements of SlyA are labelled. The DNA molecule is shown as a white stick representation. (c) Close-up view of the interactions between SlyA and DNA. The side-chains of Leu-63, Val-64, Arg-65, Leu-67, Leu-70, Arg-86 and Lys-88 are shown as yellow sticks. (d) Close-up view of the dimerization interface of two molecules. The side-chains of Leu-12 and Leu-126 of two molecules (orange and green molecules) are shown as yellow and cyan sticks, respectively.
purified and subjected to cross-linking experiments with DSS. As assessed by SDS-PAGE, the apparent molecular size of DSS cross-linked wild-type His<sub>6</sub>-SlyA (20.7 kDa, with a 40 aa N-terminal His<sub>6</sub> tag) is 42 kDa, which is approximately the expected size of 41.4 kDa for dimeric His<sub>6</sub>-SlyA (Fig. 5c). However, mutant SlyA protein L12A failed to form a detectable cross-linked dimer (Fig. 5c). While the L126A substitution mutation significantly affected the levels of expression of ssrA in Salmonella, the mutant protein showed reduced but detectable dimer formation upon DSS

Fig. 3. DNA-binding properties of substitution mutants of the SlyA protein. (a) Whole-cell lysates corresponding to 1 x 10<sup>7</sup> bacteria isolated from the S. enterica serovar Typhimurium slyA mutant strain harbouring a chromosomal ssrA-lacZ fusion gene (SH122) with plasmids pBAD-slyA and the derivatives of pBAD-slyA were subjected to Western blot analysis with anti-Penta.His antibody. Salmonella strain SH122 harbouring vector pBAD-HisA (pBAD) was used as a negative control. (b) Comparison of the functional activity of the wild-type SlyA and each mutant. The activity of the ssrA promoter was estimated by measuring the β-galactosidase activity. (c) Binding of purified wild-type SlyA (wt) and point-mutant SlyA proteins (L63A, R65A, L70A, R86A and K88A) to DNA fragment a containing the putative SlyA-binding site of the ssrA regulatory region (see Fig. 1c). The DNA fragment was incubated without protein (lane 1), with increasing amounts of the purified SlyA protein (6, 9 and 12 μM; lanes 2–4), or with 1.5 mM BSA (lane 5). The corresponding molecular masses are indicated on the left.
cross-linking followed by SDS-PAGE (Fig. 5c). In contrast, eight SlyA mutant proteins that were able to promote ssrA transcription were observed in the dimer form of each purified protein (data not shown). Furthermore, these two mutant SlyA proteins, L12A and L126A, abolished binding to the ssrA promoter (data not shown). These results suggest that at least amino acid residue Leu-12 (within α1-helix) is required for SlyA to form a dimer and Leu-126 (within α6-helix) for efficient dimerization.

SlyA mutant derivatives fail to restore the ability of a slyA mutant strain to induce Sifs and F-actin meshwork formation in vivo

In line with the reduction in SPI-2 expression by mutation of slyA, a slyA mutant strain in infected cells was unable to induce Sifs and intracellular F-actin meshwork formation (Linehan et al., 2005), phenotypes which are dependent on SPI-2 TTSS, and both phenotypes are required for intracellular survival and replication by Salmonella (Waterman & Holden, 2003). To examine whether mutant SlyA proteins would be able to restore the induction of Sif formation and actin reorganization during intracellular replication, HeLa cells infected with Salmonella strains carrying plasmids expressing the wild-type and mutant SlyA proteins were fixed and subsequently labelled with an anti-LAMP-1 antibody to reveal Sifs and with Texas red-phalloidin to detect F-actin. In these experiments, the ssrA mutant was used as a negative control. In addition, to confirm that Sif formation and actin reorganization are dependent on SPI-2 TTSS function, we used the SPI-2 ssaV null mutant, which lacks an essential structure component of SPI-2 TTSS. At 20 h after bacterial invasion, approximately 65% of the cells infected with wild-type bacteria contained Sifs, whereas Sifs were detected in less than 1% of the cells infected with SPI-2 mutant strains carrying mutations in either ssrA or ssaV. Less than 1% of the cells infected with the slyA mutant strain contained Sifs (Fig. 6a). In addition, confocal microscopy showed a strong co-localization of F-action and LAMP-1 around the wild-type bacteria (approx. 80% of the bacteria), but cells infected with the ssaV mutant strain did not show such co-localization (~10% of the bacteria). In contrast to the lack of an association in the case of the ssaV mutant strain, a close association between F-actin and LAMP-1 was observed in ~50% of the ssrA mutant bacteria. This leaky phenotype was probably due to low levels of expression of SsrB, as was also seen in the ssrA null mutant strain (Feng et al., 2003, 2004). A similar pattern of co-localization was observed in the case of the slyA mutant strain (Fig. 6b), thus confirming the results of previous studies (Linehan et al., 2005). Expression of the wild-type SlyA from a plasmid restored the ability of the mutant strain to induce Sif formation and

**Fig. 4.** Sequence alignment of amino acid residues of the winged-helix region of the MarR/SlyA family. The numbers indicate the position of the amino acid residues. The proteins used for the alignment were from Salmonella enterica serovar Typhimurium (SlyA), Yersinia enterocolitica (RovA), Erwinia carotovora (Hor), Serratia marcescens (Rap), Escherichia coli (MarR), Enterococcus faecalis (SlyA-Ef), Butyrivibrio fibrisolvens (CinR), Erwinia chrysanthemi (PecS), Pseudomonas aeruginosa (MexR) and E. coli (MprA).

**Fig. 5.** Dimerization properties of the substitution mutants of the SlyA protein. (a) Whole-cell lysates corresponding to 1 x 10^7 bacteria isolated from the S. enterica serovar Typhimurium slyA mutant strain harbouring chromosome ssrA-lacZ fusion gene (SH122) with pBAD-slyA and the derivatives of pBAD-slyA were subjected to Western blot analysis with anti-Penta.His antibody. (b) Comparison of the functional activity of the wild-type SlyA and that of each mutant. (c) Cross-linking of SlyA and its mutant derivatives (L12A and L126A) with DSS. The positions of molecular mass markers are shown on the left.
The regulatory protein SlyA plays major roles in Salmonella virulence, resistance to oxidative stress and resistance to antimicrobial peptides (Buchmeier et al., 1997; Daniels et al., 1996; Kaneko et al., 2002; Libby et al., 1994; Shi et al., 2004; Watson et al., 1999). Recently, it was reported that an ssrA-lacZ fusion gene in E. coli is activated in the presence of SlyA (Feng et al., 2004). In addition, using cDNA microarray analysis, a number of genes in the SlyA regulon have been identified, including the ssrAB locus, which regulates the expression of genes encoded by SPI-2 (Navarre et al., 2005). Consistent with these data, it has been demonstrated that the inability of a Salmonella slyA mutant strain to proliferate in phagocytes and host tissues is due to the loss of SPI-2 function (Linehan et al., 2005). In this study, to analyse the molecular mechanism of SlyA in Salmonella virulence, we characterized SlyA function in the context of the ssrA regulatory process. We demonstrated that SlyA acts directly on the ssrA promoter and that the mutation in SlyA that disables DNA-binding activity resulted in the reduced expression of ssrA, in turn leading to a loss of SPI-2 function.

We have shown that SlyA controls ssrA transcription directly by binding to the ssrA promoter. Competitive DNA-binding assays with purified His6-SlyA protein revealed that SlyA interacts with nucleotides within the region −100 to −13 of the ssrA promoter, which is essential for the formation of the SlyA–DNA complex. In this region, we found a 7 bp inverted repeat sequence, TTATTATTAATAA (positions −33 to −19 of the ssrA promoter region), which serves as the putative SlyA binding site, and which resembles the putative consensus SlyA-binding motif (TTAGCAAGCTAA) described for the slyA promoter in vitro (Stapleton et al., 2002). Interestingly, although additional experiments including DNA footprinting assays will be required to provide further evidence of the sequence-specific binding of SlyA, the location of the SlyA-binding site appears to be a region within the −35 and −10 promoter sequence, which is an unusual site for a transcriptional activating protein. However, SlyA has also been shown to have binding sites downstream of the transcriptional start site in the pagC and ugtL promoters that are required for gene transcription (Navarre et al., 2005; Shi et al., 2004).

Members of the MarR/SlyA family both activate and repress the expression of several genes. The data presented for SlyA and its homologue, RovA from Yersinia, suggest that the regulatory proteins of this family may positively regulate genes by acting as derepressors (Heroven et al., 2004; Wyborn et al., 2004). For example, E. coli hlyE (also known as slyA and sheA), which encodes a novel pore-forming toxin, is regulated positively by the global transcriptional factors FNR (fumarate and nitrate reduction) and CRP (cAMP receptor protein) and by SlyA, and negatively by the nucleoid-structured protein H-NS (Wyborn et al., 2004). Both H-NS and SlyA interact directly with the hlyE promoter region and occupy the same sequence that overlaps the binding site for the global transcriptional factors, suggesting that SlyA functions as a derepressor that competes with H-NS for the binding site within the hlyE promoter. Similarly, in the case of ssrA, the binding of SlyA might change the local nucleoprotein structure of the chromosome by competing with the negative action of repressors (such as H-NS), and enabling activation of ssrA by response regulators OmpR and SsrB.

SlyA of S. enterica has been identified as a member of the MarR/SlyA family, based on the similarity of its amino acid sequence to that of other members of this family (Ludwig et al., 1995; Thomson et al., 1997). Although the amino acid sequence similarity of this SlyA is relatively low, we demonstrated that the structure of this SlyA is very similar to the structures of the other MarR family proteins. The crystal structures of this protein family (which includes

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**DISCUSSION**

The regulatory protein SlyA plays major roles in Salmonella virulence, resistance to oxidative stress and resistance to antimicrobial peptides (Buchmeier et al., 1997; Daniels et al., 1996; Kaneko et al., 2002; Libby et al., 1994; Shi et al., 2004; Watson et al., 1999). Recently, it was reported that an ssrA-lacZ fusion gene in E. coli is activated in the presence of SlyA (Feng et al., 2004). In addition, using cDNA microarray analysis, a number of genes in the SlyA regulon have been identified, including the ssrAB locus, which regulates the expression of genes encoded by SPI-2 (Navarre et al., 2005). Consistent with these data, it has been demonstrated that the inability of a Salmonella slyA mutant strain to proliferate in phagocytes and host tissues is due to the loss of SPI-2 function (Linehan et al., 2005). In this study, to analyse the molecular mechanism of SlyA in Salmonella virulence, we characterized SlyA function in the context of the ssrA regulatory process. We demonstrated that SlyA acts directly on the ssrA promoter and that the mutation in SlyA that disables DNA-binding activity resulted in the reduced expression of ssrA, in turn leading to a loss of SPI-2 function.

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MarR, MexR, SarR, SlyA-Ef, AphA and OhrR) revealed that the protein forms a homodimer, with each subunit possessing a winged-helix DNA binding motif, which is required for DNA binding (Alekshun et al., 2001; De Silva et al., 2005; Hong et al., 2005; Lim et al., 2002; Liu et al., 2001; Wu et al., 2003). Furthermore, winged-helix proteins characteristically bind to DNA sequences containing a palindromic or pseudopalindromic structure. For example, the binding site of MarR contains two inverted 5 bp sequences separated by 2 bp (Egland & Harwood, 1999); moreover, in the case of MexR, the 5 bp inverted repeat sequences are separated by 5 bp (Evans et al., 2001). The binding site for SlyA contains a 5 bp inverted repeat separated by 2 bp at the slyA promoter, although there is variation within DNA sequences among the different promoters.

The structure of OhrR bound to the ohrA operator complex reveals the DNA-binding mechanism for a regulator in the MarR family (Hong et al., 2005). When OhrR binds to DNA, the α4 helix of the helix–turn–helix domain makes contact with the major groove, and the wing, composed of strands β2 and β3 and their connecting loop, interacts with the minor groove. In addition to the winged helix–turn–helix motif, the helix–helix DNA-binding element interacts with the phosphate backbone of the DNA (Hong et al., 2005). Thus, OhrR dimer locates the target DNA by an extended wing, a helix–turn–helix motif and the helix–helix interaction motif. In our model of the SlyA structure in complex with DNA, the wing is packed alongside the DNA, spanning the phosphate backbones of both strands on either side of the minor groove, and helix α4 is thus expected to be involved in interactions with the major groove. Since the similarity among MarR/SlyA family proteins appears to be limited to the DNA-binding domains, the requirement of a helix–helix motif for DNA binding is unclear. Further structural studies of SlyA in the presence of DNA will be required to fully understand the nature of the interaction between SlyA and a target DNA.

The reported structures of MarR family proteins show that the dimerization domain includes α-helices in the N-terminal and C-terminal regions of each monomer (Alekshun et al., 2001; De Silva et al., 2005; Hong et al., 2005; Lim et al., 2002; Liu et al., 2001; Wu et al., 2003). The protein–protein interaction is primarily mediated by a number of hydrophobic interactions, and is further stabilized by hydrogen bonds and salt-bridge pairs. Likewise, in the predicted SlyA dimer structure, the dimer interface appears to be present between an N-terminal α-helix, α1, and a C-terminal α-helix, α6. In line with this model, the L12A mutation located in α-helix α1 and the L126A mutation located in α-helix α6 were shown to reduce the ability of the protein to dimerize. Whereas the effects of L126A mutation on dimerization were quite weak, these mutations exerted significant effects on both the expression of sssA and SPI-2 function in infected HeLa cells. In addition, the mutant SlyA protein, which cannot form a dimer, is incapable of binding to DNA. Thus, the amino acid residues that are critical for the formation and/or maintenance of the dimer structure are also important for SlyA function as a transcriptional regulator.

More recently, the structure–function analysis has been reported of Yersinia RovA, which is required for full virulence and efficient colonization by Yersinia (Revell & Miller, 2000; Tran et al., 2005). Functionally important domains of RovA were determined by random mutagenesis and terminal deletions. Consistent with our study, Tran et al. (2005) identified several substitutions in the winged-helix domain in the centre of the molecule, which is essential for DNA binding, and isolated amino acid changes within both termini that reduced dimer formation. In addition, they proposed a structure model for the RovA protein, which is very similar to that of SlyA in this study. However, several functional amino acids of RovA required for efficient DNA binding were different from those of SlyA. This difference might be due to diverse DNA targets of the two proteins, showing that regulatory proteins belonging to the SlyA family are highly adapted for species-specific regulation.

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Molecular and cellular functions of the S. typhimurium slyA gene


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