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DNA recognition by the *Salmonella enterica* serovar Typhimurium transcription factor SlyA

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Summary. The *Salmonella* regulatory protein SlyA is implicated in virulence, survival in macrophages and resistance to oxidative stress and anti-microbial peptides. SlyA is a member of the MarR family of winged-helix transcription factors. Systematic mutational analysis of the SlyA operator sequence and of the predicted DNA-binding region of SlyA shows that no single base pair in the palindromic SlyA operator sequence is essential for DNA binding, and identifies amino acid residues required to allow SlyA to recognise DNA. Combining the structure-function studies described here and elsewhere with the structures of MarR family proteins suggests a possible model for regulation of SlyA binding to DNA. [**Int Microbiol** 2008; 11(4):245-250]

Key words: *Salmonella* · gene regulation · DNA binding · SlyA · MarR · OhrR

Introduction

Appropriate regulation of gene expression is essential for bacterial pathogens to adapt to a changing host environment. SlyA is a transcription factor that is required for survival of *Salmonella enterica* serovar Typhimurium (*S. typhimurium*) in macrophages and for resistance to oxidative stress and antimicrobial peptides [2,6,8,16]. *Salmonella typhimurium slyA* mutants are severely attenuated for virulence in mice by a variety of infection routes [3,6], although SlyA does not appear to be required for establishing or maintaining enteritis in cattle [21]. Proteomic and transcriptomic analyses have shown that SlyA regulates the expression of a large number of genes, many of which are predicted to encode membrane, periplasmic or secreted proteins [12,17,18].

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SlyA is a member of the MarR family of dimeric, winged-helix transcription regulators. The crystal structures of several family members are now available [1,5,7,11,24]. *Salmonella typhimurium* SlyA is a dimeric protein that recognises five sites within the *slyA* promoter that have DNA sequences related to the 12-bp partially palindromic sequence TTAGCAAGCTAA [18]. SlyA binding sites with similar DNA sequences have been identified in other promoters, including those of the *ugtL*, *pagC*, *mig-14* genes of *Salmonella*, and the *hlyE* gene of *Escherichia coli* [12,16,25]. Binding of SlyA at target promoters causes activation or repression of gene expression. SlyA-mediated activation is thought to occur by antagonising the action of repressors such as H-NS, rather than by directly recruiting RNA polymerase, and has been implicated in allowing expression of horizontally acquired genes that are HNS-silenced [9,14,25]. Although it is known that *Salmonella slyA* expression is enhanced in stationary phase and in macrophages [2], it is not known whether SlyA activity is controlled entirely by changes in intracellular concentration or whether SlyA activity can also be modulated by the binding of a ligand. SlyA was first identified by its ability to confer a haemolytic phenotype on *E. coli* K-12 [6]. Subsequently this has shown to be caused by activation of expression of *hlyE*, a gene encoding a pore-forming toxin capable

of lysing red blood cells [10]. SlyA activates *hlyE* expression by antagonising H-NS-mediated repression [9]. Thus, the inability of SlyA variants to confer a haemolytic phenotype on *E. coli* K-12 could be used to identify loss of function mutants.

During the current work a structure-function analysis of *S. typhimurium* SlyA was published [13]. Okada et al. presented a model of the SlyA:DNA complex and used this to guide a site-directed mutagenesis study to identify amino acid residues required for DNA binding and dimerisation [13]. Nine SlyA variants were shown to be inactive in vivo and in vitro, including seven amino acid residues (Leu-63, Val-64, Arg-65, Leu-67, Leu-70, Arg-86 and Lys-88) in the winged-helix region and two (Leu-12 and Leu126) in the dimer interface [13]. Here the effects of random and targeted mutagenesis of the *slyA* coding region and the SlyA operator sequence confirm and extend previous observations [13].

Materials and methods

Oligonucleotides and synthetic SlyA-binding DNA fragments. The 46-mer oligonucleotide S_{CON}f was designed to contain the SlyA-binding site ($^{-6}$T^{-5}T^{-4}A^{-3}G^{-2}C^{-1}A^{1}A^{2}G^{3}C^{4}T^{5}A^{6}A, base positions indicated by numbering from –6 to 6) positioned between –35 and –10 RNA polymerase binding elements (Table 1). Oligonucleotides $S_{-6/6}$f to $S_{-1/1}$f are variants of S_{CON}f with two bases changed in the same relative position in both half sites of the palindrome (e.g., –6 and 6). S_{NEG}f was a control with no SlyA binding site based on the spacer region of the semi-synthetic promoter pFF-41.5 [23]. Each of these were annealed with complementary oligonucleotides (designated by r), by heating to 94ºC for 5 min and then slowly cooling to produce double-stranded DNA fragments with *Bam*HI- and *Hin*dIII–compatible ends. Oligonucleotides were synthesised by Sigma (Haverhill, UK).

Bacterial strains, plasmids and microbiological methods. Relevant characteristics of bacterial strains and plasmids used are given in Table 1-SI. Bacteria were grown in Luria broth (tryptone 10 g/l; yeast extract 5 g/l; NaCl 10 g/l) at 37ºC. This medium was supplemented with ampicillin (100 μg/ml) when appropriate. Standard methods for manipulation of DNA were followed [15]. To construct pGS1329, a fragment containing the *slyA* gene and promoter region was amplified and isolated as a 715 bp product by PCR using *Salmonella typhimurium* LT2 genomic DNA as template and primers (JGS1 and JGS2), that contain unique *Bam*HI and *Eco*RI restriction sites to facilitate cloning into pUC118. Plasmid pGS1329 was used as the template (~1 ng per reaction) to amplify the *slyA* promoter and coding regions using the oligonucleotides JGS1 and JGS2 (1 μM) in error-prone PCR reactions to create libraries of random *slyA* mutants. The reaction conditions were: 40 cycles of 95°C for 0.5 min, 50°C for 1.5 min, 72°C for 5 min, in the presence of 3 mM $MgCl_2$ and 2.5 U *Taq* DNA polymerase. The amplified products were ligated into pBluescript II SK and propagated in *E. coli* JM109. Plasmids carrying loss of function *slyA* alleles were selected by the failure to confer a haemolytic phenotype on blood agar (Columbia blood agar base with 5% defibrinated horse blood, TCS Microbiology) containing 100 μg/ml ampicillin. Non-haemolytic colonies were selected, the plasmids were recovered and the *slyA* genes were sequenced. Some of these genes encoding altered SlyA proteins were selected, amplified by PCR and ligated into pGEX-KG as *Bam*HI-*Sal*I fragments (Table 1) to allow overproduction of GST-SlyA fusion proteins and isolation, by thrombin cleavage, of the corresponding SlyA variants for analysis by electromobility shift assays (EMSA).

Protein purification and site-directed mutagenesis. SlyA was overproduced using a GST-SlyA fusion (pGS1482) in *E. coli* BL21/λDE3 as described previously [18]. The SlyA protein was released from the fusion by on column thrombin cleavage; the GST was retained on the GSH Sepharose column. Plasmid pGS1482 was also used as the template for site-directed mutagenesis using the Stratagene Quikchange system. Amino acid residues within the predicted SlyA DNA-binding domain were replaced by alanine using appropriate mutagenic oligonucleotides. The authenticities of the plasmids created were checked by DNA sequencing and the altered SlyA proteins were overproduced and isolated as described for unaltered SlyA [18]. Gel filtration (Shodex KW803 300 × 4.5 mm) was used to determine the oligomeric states of SlyA variants that failed to bind DNA. The column was equilibrated with 10 mM Na_2HPO_4, 1.75 mM KH_2PO_4, 2.7 mM KCl and 135 mM NaCl, pH 7.0, and calibrated with the following protein standards: myosin (220 kDa), bovine serum albumin (66 kDa), ovalbumin (43 kDa), carbonic anhydrase (29 kDa) and myoglobin (17 kDa).

Electromobility shift assays and quantification. To compare the affinity of the altered SlyA binding sites ($S_{-6/6}$–$S_{-1/1}$) with the SlyA consensus (S_{CON}), 20 μg S_{CON} DNA was radioactively labelled using Klenow fragment and 20 μCi [α^{32}P]dATP for use in competitive gel shift assays. In a 20 μl reaction volume, 0.3 μM labelled S_{CON} (S_{CON}*) was incubated at room temperature for 15 min with 2 μg of SlyA protein, 2 μl of 10× binding buffer (100 mM Tris-HCl pH 7.5, 10 mM EDTA, 50 mM DTT, 50% glycerol, 100 mM NaCl, 10 mM $MgCl_2$), and up to 20 μM of unlabelled "test" fragments ($S_{-6/6}$ to $S_{-1/1}$) as indicated. Reactions were separated using a 4% acrylamide, 1× TBE (100 mM Tris, 120 mM boric acid, 10 mM EDTA pH 8.0) gel with a 1x TBE running buffer. Gels were dried on Whatman paper and subjected to autoradiographic analysis. The intensity of bands in the free DNA position was quantified using Imagemaster software. Similar electromobility shift assays were used to analyse interaction of SlyA variants with radiolabelled *slyA* promoter DNA, amplified from pGS1384 [18] using oligonucleotides VN7 and VN8 (Table 1-SI).

Results

Effects of symmetrical changes in the SlyA operator sequence on SlyA binding. Stapleton et al. [18] identified the palindromic SlyA-binding sequence $^{-6}$T^{-5}T^{-4}A^{-3}G^{-2}C^{-1}A^{1}A^{2}G^{3}C^{4}T^{5}A^{6}A (base positions identified by numbering from –6 to 6) within the SlyA promoter region. To determine the relative importance of base pairs in this motif for SlyA binding, variant DNA fragments with base substitutions at symmetric positions were synthesised (S_{CON} contains the unaltered sequence, $S_{-6/6}$ has substitutions at positions –6 and 6, $S_{-5/5}$ has substitutions at positions –5 and 5 etc., Table 1-SI), for use in electromobility shift assays (EMSA). A seventh fragment was synthesised (S_{NEG}) that lacked a SlyA binding site. Radioactively-labelled S_{CON} DNA (S_{CON}*, unaltered SlyA binding site, 0.3 μM) was incubated with SlyA (3 μM) to form a complex. Unlabelled S_{CON} or S_{NEG} DNA was then added to compete for the SlyA in the assay. Upon addition of the unlabelled competitor DNA the mobility of the complex initially increased, presumably due to the

relief of non-specific SlyA binding. Subsequently, the amount of SlyA:S_{CON}* complex decreased upon addition of increasing amounts of unlabelled S_{CON}, whereas S_{NEG} DNA competed less effectively (Fig. 1A). Similar experiments with the altered SlyA binding sites ($S_{-6/6}$, $S_{-5/5}$, $S_{-4/4}$, $S_{-3/3}$, $S_{-2/2}$, and $S_{-1/1}$) were done and the amount of S_{CON}* released was quantified. As expected the unchanged SlyA consensus (S_{CON}) was the best competitor; however the altered SlyA binding sites also successfully competed with S_{CON}*, but in all cases higher concentrations were required compared to S_{CON} (Fig. 1B). From the data shown in Fig. 1 the apparent K_d values for each competitor DNA could be estimated. This showed that the competitor DNAs fell into three classes. The first class was unlabelled S_{CON} with a $K_{d(app)}$ of ~0.5 μM; the second class contained $S_{-1/1}$, $S_{-2/2}$, and $S_{-3/3}$ with $K_{d(app)}$ values in the range 2.0–2.6 μM; the third class contained $S_{-4/4}$, $S_{-5/5}$, and $S_{-6/6}$ with $K_{d(app)}$ values in the range 4.4–5.4 μM. Thus, the least effective competitors were those with mutations at positions –6/6, –5/5, and –4/4, suggesting that these positions contribute most to recognition of these DNA sequences by SlyA.

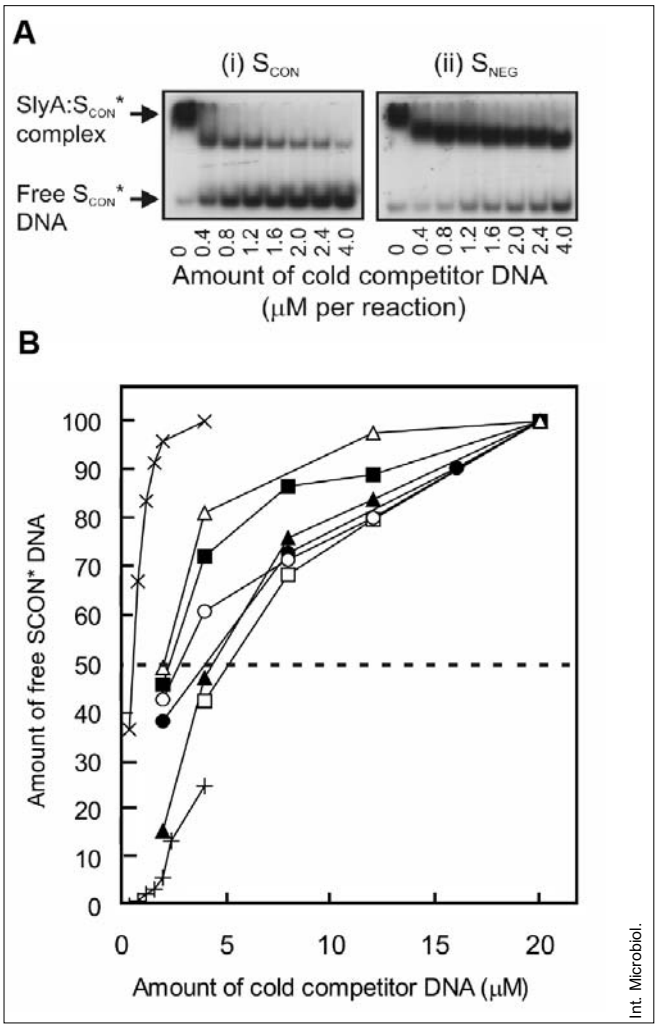

Fig. 1. Competition between a radiolabelled consensus SlyA binding site (S_{CON}* TTAGCAAGCTAA) and unlabelled mutated sites for SlyA in EMSAs. (**A**) Representative competitive ESMAs with increasing concentrations of cold competitor DNA as follows (i) S_{CON} (TTAGCAAGCTAA); (ii) S_{NEG} (no SlyA binding site CCCCCTCACTTC). The S_{CON}*:SlyA complex was formed from 0.4 μM of radiolabelled S_{CON} DNA (S_{CON}*) and 3 μM SlyA. (**B**) The intensities of the free S_{CON}* DNA were measured and the percentage of free S_{CON}* DNA was plotted against the amount of unlabelled S_{CON} (×), $S_{-6/6}$ (closed circles), $S_{-5/5}$ (open squares), $S_{-4/4}$ (closed triangles), $S_{-3/3}$ (open circles), $S_{-2/2}$ (closed squares), $S_{-1/1}$ (open triangles), and S_{NEG} (+) DNA added (for sequences of the SlyA binding sites in the competitor DNAs see Table 1). The dashed line shows point at which approximately 50% of S_{CON}* DNA was released from the S_{CON}*:SlyA complex. The data shown are typical of those obtained from at least two experiments.

Identification of SlyA amino acid residues essential for DNA recognition. Error-prone PCR was used to create a library of mutant *slyA* genes that were expressed from the *slyA* promoter in pBluescript and screened for the ability to confer a haemolytic phenotype on *E. coli* K-12. This approach yielded six loss of function *slyA* mutants with multiple amino acid replacements (SlyA-W16R/E105G, SlyA-L29S/R85C, SlyA-L37W/L52F/E97D, SlyA-H38R/D68G, SlyA-K54N/E94V/M103L, SlyA-R85H/I107V/K132E), and seven with single amino acid substitutions (SlyA-V13E, SlyA-N39D, SlyA-A53V, SlyA-V64I, SlyA-R85H, SlyA-I100N, SlyA-L126R). Only the SlyA variants with single amino acid substitutions were studied further. Western blotting using anti-SlyA serum revealed that all the plasmid encoded singly substituted proteins were expressed to similar levels to that of plasmid encoded unaltered SlyA (not shown). Alignment of the SlyA and MarR primary structures allowed the 3-D locations of the amino acids in SlyA that are required to confer a haemolytic phenotype on *E. coli* K-12 to be predicted using the MarR crystal structure [1]. This revealed that loss of function was associated with amino acid replacements in the predicted SlyA dimerisation (V13E, L126R) and DNA-binding (A53V, V64I and R85H) regions. Based on the MarR structure, in the SlyA dimer, Val13 and Leu126 of one protomer are predicted to be close to the corresponding residues in the other protomer. The replacement of a non-polar amino acid (Val/Leu) by a charged residue (Glu/Arg) could thus produce a charge clash altering the conformation of the SlyA dimer, or preventing its formation. Isolated SlyA-V13E and SlyA-L126R proteins failed to bind target DNA in ESMAs, suggesting that correct dimerisation is essential for DNA-binding (not shown). The SlyA variant SlyA-R85H also failed to bind DNA, whereas SlyA-V64I formed a relatively low affinity complex (Fig. 2A). The remaining two SlyA variants (SlyA-N39D and SlyA-I100N) had amino acid replacements in regions outside

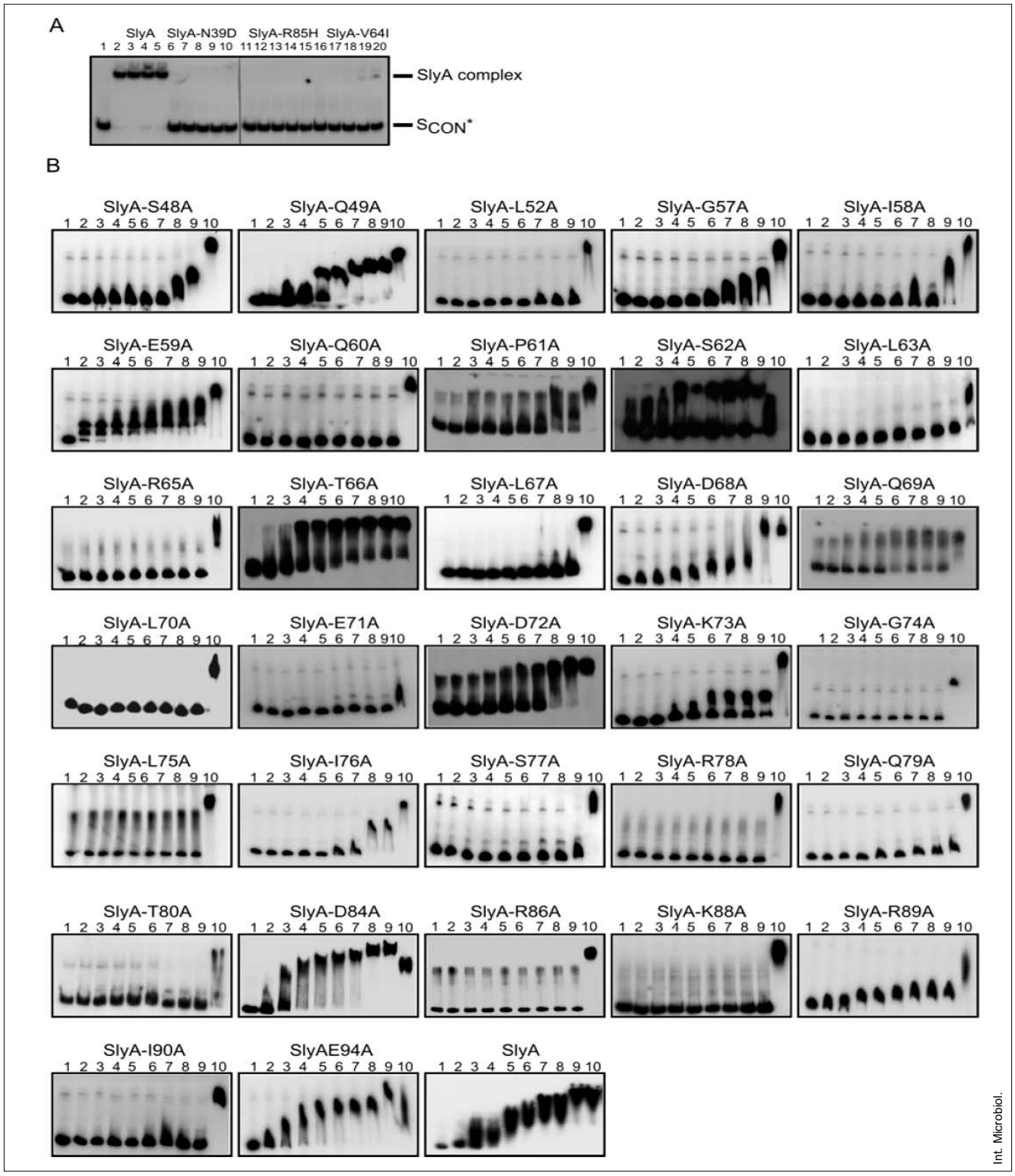

Fig. 2. Electromobility shift assays with SlyA variants. (**A**) SlyA variants identified by screening error-prone PCR libraries on blood agar for non-haemolytic variants. Lanes 1, 6, 11, 16, no protein; lanes 2–5, SlyA at 23, 46, 69 94 μM. Lanes 7–10, SlyA–N39D at 23, 46, 69, 94 μM; lanes 12–15, SlyA-R85H at 13, 27, 40, 54 μM; lanes 17–20, SlyA-V64I at 9, 19, 28, 37 μM. The target DNA was S_{CON}* (see Fig. 1 and Table 1). (**B**) SlyA variants generated by site-directed mutagenesis. ESMA in the presence of increasing concentrations of the indicated SlyA variants. The target DNA was the *slyA* promoter (P*slyA*). Lane 1, no protein; lane 10, wild-type SlyA 2 μM; lanes 2–9 variant SlyA at 0.4 μM, 0.8 μM, 1.2 μM, 1.6 μM, 2.0 μM, 3.0 μM, 4.0 μM and 5.0 μM final concentration. The data shown are typical of those obtained from at least two experiments.

those involved in DNA-binding or dimerisation. However, these residues are predicted to be close to each other in 3-D space and the two helices in which they are located link the N-/C-terminal regions of the SlyA protein to the DNA-binding region. SlyA-N39D failed to bind target DNA (Fig. 2A) and it is possible that alterations in these linking helices impair the correct positioning of the DNA-binding domains.

Using an alignment of the primary structures of MarR and SlyA to delimit the predicted DNA binding region of SlyA, mutant *slyA* alleles were created by replacing single amino acids between residues 48 to 94 by Ala. Increasing concentrations of altered SlyA proteins were incubated with P*slyA* as the target DNA (Fig. 2B). The *slyA* promoter contains five SlyA binding sites and under the conditions used here the apparent affinity of SlyA (0.4-0.8 μM) for the promoter was similar to that previously reported [18]. The results with the SlyA variants indicated that replacement of amino acids at position 48, 49, 57, 58, 59, 61, 62, 66, 68, 69, 72, 73, 76, 84, 89 and 94 did not abolish the DNA-binding ability of SlyA, and thus these amino acids are not essential for SlyA:DNA interactions. Placing alanine residues at any of the following positions 52, 60, 63, 65, 67, 70, 71, 74, 75, 77, 78, 79, 80, 86, 88 and 90 significantly inhibited DNA-binding under the conditions tested, suggesting that these amino acids are important for SlyA:DNA interactions. All of the SlyA variants that were created by site-directed mutagenesis and failed to bind DNA were soluble. Furthermore, all but two (SlyA-L52A and SlyA-L63A, which eluted as higher molecular weight oligomers) eluted from a gel filtration column as dimers (not shown), suggesting that the failure of these proteins to bind DNA is not caused by a problem in assembling the SlyA dimer, and is more likely due to the inability to form optimum protein:DNA contacts.

Discussion

The work reported here provides a better understanding of structure-function relationships in the SlyA:DNA complex by showing the effects of mutagenesis of the target DNA and the SlyA protein. Replacement of symmetrically related base pairs within the 12-bp SlyA binding site previously proposed [18] revealed that no single position was essential for SlyA binding, but that all positions contribute to SlyA recognition to some degree. However, the data suggest that the most important bases pairs for recognition of DNA by SlyA are located at the 5′ and 3′ regions (bold) of the consensus site **TTA**G-CAAGC**TAA**. Alignment of SlyA binding sites identified by footprinting of the *pagC*, *mig-14*, *ugtL*, *hlyE* and *slyA* promoters [9,12,16,18,25] supported this suggestion, because of the 15 SlyA sites in these promoters, positions –6 (T 12/15), –5 (T 10/15), –4 (A 10/15), –3 (G 10/15), –1 (14/15), 5 (A 14/15), and 6 (A 12/15) were most conserved; at the other positions the degree of conservation was 7/15 or worse. The recognition that the bases at positions –6/6, –5/5, and –4/4 are most important for SlyA binding in vitro should help to delimit the SlyA regulon by facilitating the use of bioinformatic tools to analyse genome sequences for genes directly regulated by SlyA.

Random and targeted mutagenesis showed that the integrity of the predicted wing region (amino acids 76–91) in the SlyA DNA-binding domain is essential for DNA binding. The structure of the MarR family member OhrR bound at its operator DNA shows that the residues of OhrR (Arg-86 and Arg-94) that interact with the minor groove of the DNA target are conserved in SlyA (Arg-78 and Arg-86). In addition, Asp-92 of OhrR which makes contacts with Arg-94 is also conserved in SlyA (Asp-84), suggesting a similar mechanism of interaction, i.e., a pyrimidine:Arg-86:Asp-84 interaction accompanied by an Arg-78 backbone contact [5].

Mutagenesis of the predicted SlyA DNA-recognition helix (α_4 amino acids 60–73) identified two surfaces required for DNA-binding. A largely hydrophobic surface consisting of Gln-60, Leu-63, Val-64, Leu-67, and Leu-70 is suggested to mediate intra-protomer interactions to maintain the tertiary structure of the helix-turn-helix domain. The abolition of DNA-binding when any of these amino acids was replaced is entirely consistent with the work of Okada et al. [13], which showed inhibition of DNA-binding and in vivo activity of SlyA by amino acid replacements at positions 63, 67 and 70. Also consistent with previous observations, the only other replacement of an α_4 amino acid residue that abolished DNA-binding was Arg-65 to Ala [13]. Arg-65 is located on the face of the α_4 helix opposite to the hydrophobic leucine residues and is predicted to be part of a hydrophilic surface consisting of Pro-61, Arg-65, Asp-68, Gln-69, Glu-71 and Asp-72. Arg-65 is conserved in 99 of the top 100 sequences returned when the amino acid sequence of the DNA-binding domain (amino acids 47–94) of SlyA is used to interrogate the NCBI Blast number database, suggesting that it makes a conserved interaction with target DNA in the SlyA group of proteins. Residue Arg-65 of SlyA corresponds to Arg-73 of MarR. In the non-DNA-binding form of MarR (the MarR salicylate complex) Arg-73 of one protomer (Asp-73) interacts with Asp-67 of the other (Asp-67′) to stabilise the relative orientations of the DNA-binding lobes of the MarR dimer [1]. In SlyA, the equivalent residue to Asp-67 is Glu-59 (conserved in 96 of the top 100 sequences). Glu-59 is not essential for DNA-binding (Fig. 2) but is a good candidate to mediate an interaction between SlyA protomers (Glu-59′:Arg-65) simi-

lar to that between Asp-67′ and Arg-73 of MarR. This suggests a plausible model for regulating the ability of SlyA to bind DNA in which SlyA interacts with DNA in a similar manner to OhrR, with a conserved interaction between the wing region and the DNA minor groove, and sequence specific interactions in the major groove mediated by amino acids in α_4 (including Arg-65 of SlyA), but that in the presence of an as yet unidentified ligand Glu-59′ might interact with Arg-65 to inhibit SlyA DNA-binding by repositioning the DNA binding lobes in a manner similar to that suggested by the MarR:salicylate complex [1,22].

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Table 1-SI. Oligonucleotides, plasmids and bacterial strains

Oligonucleotide,* strain or plasmid	Relevant characteristics	Source or reference
S_{CON}f	GATCCTTCTTTGACATCTTAGCAAGCTAATGCTATAATTCTGATAA	This work
$S_{-6/6}$f	GATCCTTCTTTGACATC***G***TAGCAAGCTA***C***TGCTATAATTCTGATAA	This work
$S_{-5/5}$f	GATCCTTCTTTGACATCT***G***AGCAAGCT***C***ATGCTATAATTCTGATAA	This work
$S_{-4/4}$f	GATCCTTCTTTGACATCTT***G***GCAAGC***C***AATGCTATAATTCTGATAA	This work
$S_{-3/3}$f	GATCCTTCTTTGACATCTTA***T***CAAG***A***TAATGCTATAATTCTGATAA	This work
$S_{-2/2}$f	GATCCTTCTTTGACATCTTAG***T***AA***A***CTAATGCTATAATTCTGATAA	This work
$S_{-1/1}$f	GATCCTTCTTTGACATCTTAGC***GG***GCTAATGCTATAATTCTGATAA	This work
S_{NEG}f	GATCCTTCTTTGACATCCCCCCTCACTCCTGCTATAATTCTGATAA	This work
S_{CON}r	AGCTTTATCAGAATTATAGCATTAGCTTGCTAAGATGTCAAAGAAG	This work
$S_{-6/6}$r	AGCTTTATCAGAATTATAGCA***G***TAGCTTGCTA***C***GATGTCAAAGAAG	This work
$S_{-5/5}$r	AGCTTTATCAGAATTATAGCAT***G***AGCTTGCT***C***AGATGTCAAAGAAG	This work
$S_{-4/4}$r	AGCTTTATCAGAATTATAGCATT***G***GCTTGC***C***AAGATGTCAAAGAAG	This work
$S_{-3/3}$r	AGCTTTATCAGAATTATAGCATTA***T***CTTG***A***TAAGATGTCAAAGAAG	This work
$S_{-2/2}$r	AGCTTTATCAGAATTATAGCATTAG***T***TT***A***CTAAGATGTCAAAGAAG	This work
$S_{-1/1}$r	AGCTTTATCAGAATTATAGCATTAGC***CC***GCTAAGATGTCAAAGAAG	This work
S_{NEG}r	AGCTTTATCAGAATTATAGCAGGAGTGAGGGGGGATGTCAAAGAAG	This work
JGS1	TTTTGAATTCAATGCTTTAGTTTTAGCC	[18]
JGS2	TTTTGGATCCCGGCAGGTCAGCGTG	[18]
VN7	TTTTGAATTCAGAATGGCGGAAAGTAAACAGATG	[18]
VN8	TTTTGGATCCTTGATGAATATTGTGCAACGTGA	[18]
E. coli BL21/λDE3	Protease deficient strain used for protein expression	[19]
E. coli DH5α	Δ*lac*, general cloning host	[15]
E. coli JM109	Used as a host to screen error prone PCR *slyA* library	[15]
pBluescript	Ap^R cloning vector	Stratagene
pGEX-KG	Ap^R GST-fusion expression vector	[4]
pUC118	Ap^R high copy-number cloning vector	[20]
pGS1329	pUC118 based *slyA* expression plasmid	This work
pGS1384	pRW50 containing the *slyA* promoter	[18]
pGS1482	pGEX-KG based *slyA* expression plasmid	[18]
pGS1951-1990	pGEX plasmids encoding SlyA variants created by site-directed mutagenesis	This work
pGS2070-2076	pGEX plasmids encoding SlyA variants created by error-prone PCR	This work

*Oligonucleotides are shown in 5′–3′ orientation. Oligonucleotides with the f designation were annealed with their partner (r) to create DNA fragments for EMSAs (see Methods). For S_{CON}f the –35 and –10 promoter elements are underlined. The SlyA binding motif is shown in bold. The bases altered in the SlyA binding site in $S_{-1/1}$ to $S_{-6/6}$ are shown in bold italics.