Interaction of the *Salmonella typhimurium* Transcription and Virulence Factor SlyA with Target DNA and Identification of Members of the SlyA Regulon*

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The SlyA protein from *Salmonella typhimurium* is a transcription factor that contributes to virulence. It is shown that a slyA mutant is attenuated in the presence of murine macrophages compared with the parent strain. Moreover, after growth in minimal medium, survival of the slyA mutant was reduced. Altered levels of flagellin (HIC), PugC, IroN, and outer membrane proteins suggest that the slyA mutation affects the surface properties of *Salmonella*. The isolated SlyA protein is a cofactor-free homodimer that recognizes five sites within the promoter region of the slyA gene. One of these sites contained a near perfect inverted repeat TTAGCAAGCTAA. The other four sites contained related sequences. Occupation of the SlyA sites in the slyA promoter prevented open-complex formation, consistent with the pattern of slyA::lacZ expression parental and slyA mutant strains. By combining the footprinting data with potential SlyA binding sites recovered from a pool of random DNA sequences, a consensus was defined and used to probe the NIH *Salmonella* unfinished genomes data base. These searches revealed the presence of consensus SlyA sites upstream of *omp*, *ispA*, *xseB*, *slyA*, and a gene encoding a protein with homology to a hemagglutinin. Accordingly, transcription of an omp::lacZ fusion was reduced in a slyA mutant. Given the difficulties in obtaining a comprehensive picture of intracellular gene expression, the definition of the DNA sequence recognized by a transcription factor (SlyA) that is essential for survival in the macrophage environment should allow a complete regulon of genes with altered expression upon exposure to macrophages to be determined once the *S. typhimurium* genome annotation is complete.

*Salmonella* serotypes can infect many animal species, causing a variety of disease states ranging from mild enteritis to severe systemic salmonellosis. The precise nature of the disease is governed by the specific combination of host and serotype. Although many genes have been shown to be involved in pathogenesis, their precise roles in the various stages of disease are often ill defined. However, it seems clear that appropriate regulation of gene expression is essential for a pathogen to adapt to a particular host environment.

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determinations were done in triplicate on at least two independent cultures) according to Miller (13).

For some phenotypic tests the slyA mutant and parental strains were grown aerobically at 37 °C in a defined medium: Tris-HCl (pH 7.2) (100 mM); K2SO4 (0.5 mM); KH2PO4 (5 mM); NaCl (5 mM); NH4Cl (5 mM); MgCl2 (10 mM); CaCl2 (0.1 mM); thiamine (1 mM); and glucose (10 mM). Samples from stationary phase cultures were removed at intervals up to 48 h, and serial dilutions were plated on L agar to estimate the numbers of colony-forming units after overnight incubation at 37 °C. To investigate the effect of the slyA mutation on secreted proteins bacteria were collected by centrifugation, and the culture supernatants were filtered through 0.2-μm filters before precipitating the secreted proteins with trichloroacetic acid (10%, w/v). The precipitated proteins were washed with acetone before being dissolved in SDS-PAGE loading buffer and separated by SDS-PAGE (12.5% gels). Outer membrane proteins were also analyzed by SDS-PAGE of outer membrane fractions prepared by sarkosyl NL30 (2% v/v) treatment of total membrane proteins. Proteins were visualized with Coomassie Brilliant Blue.

Standard methods for the manipulation of DNA were followed (14).

### Table I

**Bacterial strains and plasmids used in this study**

| Strain or plasmid | Relevant characteristics | Source or reference |
|-------------------|--------------------------|---------------------|
| JM109             | endA1, recA1, gyrA96, thi, hisD17 (r6K-, mcrA), relA1, supE44, Δlac-proAB, ΔF' traD96, proAB, lacIqZM15I | Promega             |
| MC1000            |                          |                     |
| JRG4385           | JM109 pGS1452           | This work           |
| ST12/75           | wild type *Salmonella typhimurium* | T. Wallis, Institute for Animal Health, Compton, UK |
| ST12/75 slyA      | slyA mutant strain, PenR | T. Wallis, Institute for Animal Health, Compton, UK |
| pGEX-KG           | multiplicity vector for cloning PCR products | Promega |
| pRW50            | a low copy, ColE1-compatible, broad host range lac reporter vector, TetR | This work |
| pGS422           | a pUC derivative containing the PFF (–41.5) promoter used to calibrate the primer extension autoradiographs | This work |
| pGS1384         | pRW50 containing the slyA promoter | This work |
| pGS1482         | pGEX-KG containing the slyA coding region | This work |
| pGS1521         | slyA promoter and coding region in pBR322, ApR | This work |

The murine macrophage-like cell line J774 was used to investigate the effects of a slyA lesion on the interaction between *S. typhimurium* and macrophages. The J774 cells were grown in Dulbecco’s minimal essential medium (DMEM) containing 10% (v/v) fetal calf serum, and 1 mM glucose. The medium was removed from the wells. The plates were then incubated for a further 30 min in the presence of 5% CO2. The *S. typhimurium* strain ST12/75 and the isogenic slyA mutant (Table I) were grown aerobically in nutrient broth for 16 h. These cultures were used as 1% (v/v) inocula for fresh nutrient broth and grown without shaking at 37 °C in the presence of 5% CO2 to an OD600 of 0.6. The bacteria were harvested, resuspended in DMEM plus 10% (v/v) BALB/c mouse serum (Harlan Sera-Lab), and incubated at 37 °C for 30 min in the presence of 5% CO2. The bacteria were washed in PBS, resuspended in DMEM, and vortexed with glass beads for 1 min to prevent clumping of the cells. The medium was removed from the macrophages and 1 x 10^8 bacteria in 200 μl of DMEM was added to the wells. The plates were incubated at 37 °C in the presence of 5% CO2 for up to 3 h. At 30-min intervals the bacterial suspensions were removed, and the wells were washed twice with PBS. The cells were fixed with 2% paraformaldehyde at 37 °C in the presence of 5% CO2 for at least 15 min. The wells were then washed with PBS. Control wells were included, without *S. typhimurium* (DMEM only added) to check for contamination, and without macrophages (bacterial suspensions only) to check there was no significant binding of bacteria to the glass coverslips. The coverslips were irrigated with PBS containing 1:50 rabbit anti-Salmonella O antibody (Becto) and incubated at 37 °C for 12 min. This was followed by 1:20 fluorescein isothiocyanate-conjugated goat anti-rabbit IgG (Sigma) plus 5% goat serum at 37 °C for 12 min. The wells were then washed with PBS. The macrophages and bacteria were counterstained with the nucleic acid stain DAPI (4,6-diamidino-2-phenylindole dihydrochloride) (Molecular Probes), diluted 1:2,500 in PBS, and incubated at room temperature in the dark for 15 min. The DAPI solution was removed, and the wells were washed with PBS. The coverslips were removed from the wells and air-dried. They were mounted onto microscope slides with Vectashield mounting fluid (Molecular Probes) and viewed at x1000 magnification using a DMRB 1000 fluorescence microscope (Leica, Germany). The total number of DAPI-stained bacteria attached to macrophages was counted; internalization of bacteria was estimated by subtracting the numbers of extracellular bacteria (identified by co-localization with fluorescein isothiocyanate) from this number. Each experiment was carried out at least in triplicate.

**GST-SlyA Overexpression and Purification**—SlyA was amplified as a GST-SlyA fusion in JRG4385. Aerobic cultures were grown at 25 °C to an OD600 of 0.6 at which point expression was induced by the addition of IPTG (100 μg ml^-1). Incubation was continued for a further 3 h when the bacteria were collected by centrifugation and used immediately or stored at −20 °C. Clarified cell-free extracts were produced by resuspending the bacteria in 5 mM Tris-HCl (pH 8.0) containing 150 mM NaCl, 2.5 mM CaCl2 and 0.1% (v/v) 2-mercaptoethanol, and lysing the cells by two passages through a French pressure cell, followed by centrifugation. The GST-SlyA fusion protein was adsorbed onto a column (1 ml liter^−1 culture) of GST-Sepharose (Amersham Biosciences) equilibrated with the resuspending buffer. The SlyA protein was released from the column by incubating the column overnight with 5 units of thrombin (Sigma) and eluting the protein in resuspending buffer.

**Protein Analysis**—Protein concentration was estimated using the Bio-Rad protein assay using bovine serum albumin as standard. Protein purity was assessed by SDS-PAGE and staining with Coomassie Brilliant Blue. The oligomeric state of SlyA was determined by native polyacrylamide gels (4, 4.5, 5, 5.5, 6, 7, 8, and 9%) containing Tris-HCl (pH 7.2) (100 mM) K2SO4 (0.5 mM); KH2PO4 (5 mM); NaCl (5 mM); NH4Cl (5 mM); MgCl2 (10 mM); CaCl2 (0.1 mM); thiamine (1 mM); and glucose (10 mM). Samples from stationary phase cultures were removed at intervals up to 48 h, and serial dilutions were plated on L agar to estimate the numbers of colony-forming units after overnight incubation at 37 °C. To investigate the effect of the slyA mutation on secreted proteins bacteria were collected by centrifugation, and the culture supernatants were filtered through 0.2-μm filters before precipitating the secreted proteins with trichloroacetic acid (10%, w/v). The precipitated proteins were washed with acetone before being dissolved in SDS-PAGE loading buffer and separated by SDS-PAGE (12.5% gels). Outer membrane proteins were also analyzed by SDS-PAGE of outer membrane fractions prepared by sarkosyl NL30 (2% v/v) treatment of total membrane proteins. Proteins were visualized with Coomassie Brilliant Blue.

Standard methods for the manipulation of DNA were followed (14).

A fragment containing the slyA promoter was amplified and isolated as a 600-bp product by PCR using *S. typhimurium* genomic DNA as the template and primers MS1 (TTTTGATGCACTGGAATTGGAATCGCCACTAG) and MS2 (TTTTGATGCACTGGAATTGGAATCGCCACTAG) containing unique BamHI and SalI restriction sites (underlined) to facilitate cloning into the expression vector pGEX-KG (15). The resulting plasmid (pGS1482) was transformed into *E. coli* JM109 to create the overproducing strain JRG4385.

A fragment containing the slyA promoter was amplified and isolated as a 242-bp product by PCR, using *S. typhimurium* genomic DNA as the template and the primers VN7 (TTTTGATGCACTGGAATTGGAATCGCCACTAG) and VN8 (TTTTGATGCACTGGAATTGGAATCGCCACTAG) containing unique EcoRI and BamHI restriction sites (underlined) to facilitate cloning into plasmid pBR322. This plasmid is referred to as pGS1384. Alterations to the promoter sequence for foot-
combinations of proteins were incubated with ~10 ng of radiolabeled DNA for 2 min at 25 °C in 10 mM Tris-HCl (pH 9.0), 50 mM KCl, and 0.1% (w/v) Triton X-100 (total incubation volume 10 µl). Where appropriate the resulting complex was then challenged with heparin (0.1 mg ml⁻¹) for 2 min at 25 °C before loading onto the gel for autoradiographic analysis.

**DNase I Footprinting**—The reactions (total volume 10 µl) contained radiolabeled PolysA (~10 ng), SlyA (2.0 and 4.0 µM), 20 mM Tris-HCl (pH 8.0), 10 mM MgCl₂, 10 mM diisothreitol, and 5% (v/v) glycerol. The mixtures were incubated for 2 min at 25 °C, followed by digestion with DNase I (1 µl of 1 unit µl⁻¹ for 15–60 s at 25 °C). Reactions were stopped by addition of 200 µl of 0.3 M sodium acetate (pH 5.2) containing 20 mM EDTA followed by phenol/chloroform extraction. The DNA was ethanol-precipitated and resuspended in 10 µl of loading buffer (80% v/v formamide, 0.1% w/v SDS, 10% v/v glycerol, 8 mM EDTA, 0.1% w/v bromophenol blue, 0.1% w/v xylene cyanol) for electrophoretic fractionation on 6% polyacrylamide-urea gels and autoradiographic analysis. Maxam and Gilbert G tracks of the DNA fragments were used to provide a calibration (16).

**Permanganate Footprinting**—The reactions (total volume 20 µl) contained PolysA (~20 ng), 10 mM Tris-HCl (pH 9.0), 50 mM KCl, 0.1% (v/v) Triton X-100, SlyA (1.2 µM), and/or RNA polymerase (1.28 units) as appropriate. The mixtures were incubated at 25 °C for 30 min, and where indicated the resulting complexes were challenged with heparin (0.1 mg ml⁻¹) for 2 min before the addition of 1 µl of 150 mM KCl, 2.5 mM CaCl₂, and 0.1% (v/v) 2-mercaptoethanol. The mixtures were incubated for a further 4 min at 25 °C. The reactions were stopped with 50 µl of 0.3 M sodium acetate (pH 5.2) containing 20 mM EDTA and 1.5 M 2-mercaptoethanol, followed by phenol/chloroform extraction. The DNA was ethanol-precipitated and resuspended in 10 µl of loading buffer (see above) for electrophoretic fractionation on 6% polyacrylamide-urea gels and autoradiographic analysis.

**Transcript Mapping**—The transcription start point of PolysA was determined by RNA extraction (17) and primer extension. Total RNA was prepared from stationary phase (24 h) *S. typhimurium* pg1384 grown aerobically in L broth. For primer extension the method of Gerischer and Durre (18) was used with 100 µg of RNA and avian myeloblastosis virus reverse transcriptase (40 units) (Transgenomic). After ethanol precipitation the cDNA was fractionated on 6% urea-polyacrylamide gels for autoradiographic analysis. The gels were calibrated using Maxam and Gilbert G tracks of PolysA and PFFI-41.5 (16).

**Selection of the SlyA Binding Site from Random DNA Sequences**—The method was based on the SELEX procedure. The following oligonucleotides were synthesized: A, 5′-TGACGAATTCACGTG-3′; B, 5′-TGACGACATCGTTTTG-3′; C, 5′-TGGACGAATTCACGTG-3′. Double-stranded DNA was generated by PCR with 1 µM oligonucleotide A as a template and 5 µM each oligonucleotides B and C as primers in 10 mM Tris-HCl (pH 9.0), 50 mM KCl, 0.1% (v/v) Triton X-100, 1 mM MgCl₂, 0.5 mM dNTPs, and 5 units of Taq polymerase. The reaction mixture was heated at 94 °C for 2 min, then for each of 12 cycles was denatured at 92 °C for 1 min, annealed at 46 °C for 1 min, extended at 72 °C for 1 min. A sample (2 µl) of the resulting mixture was used as a template for a further amplification, and the two reactions were pooled.

**E. coli** strain JRG4385 was grown aerobically at 25 °C to *A*₉₀₀nm ~0.6. Expression of GST-SlyA was induced by the addition of 100 µg ml⁻¹ IPTG, and the cultures were incubated for a further 3 h. The bacteria were collected, resuspended in 5 mM Tris-HCl (pH 8.0) containing 150 mM NaCl, 2.5 mM CaCl₂, and 0.1% (v/v) 2-mercaptoethanol, and lysed by two passages through a French pressure cell. The extract was then clarified by centrifugation. Crude extract containing GST-SlyA was then mixed with 0.1 ml GSH-Sepharose (Amersham Biosciences). A column was made with 50 µl of the mixture, which was washed with 5 column volumes of binding buffer (10 mM Tris-HCl (pH 9.0), 50 mM KCl, 0.1% (v/v) Triton X-100). The pooled PCR products were passed through the column followed by washing with 5 column volumes of binding buffer. Bound DNA was eluted with 1 column volume of 3 M sodium acetate, and ethanol precipitated. The DNA was resuspended in 10 mM Tris-HCl (pH 8.5) and used as the template for five more PCRs (using oligonucleotides B and C as primers), which were pooled and used for further rounds of selection on GST-SlyA columns. After a total of three rounds of selection the amplified DNA was cloned into pGEM-T Easy Vector System I (Promega). After transformation into *E. coli* strain JM109, the plasmids were recovered, and the cloned regions were sequenced and analyzed. The same protocol was used with columns prepared with glutathione-S-transferase (GST) only to ensure that the DNA recovered was interacting specifically with SlyA and not GST or the column matrix.
were similar (not shown). However, survival of the slyA strain, as judged by the number of colony-forming units remaining over a 48-h period, was dramatically reduced compared with the parent (Fig. 2a). Investigation of the amount of protein present in culture supernatants of the parent and slyA strain grown in the Tris-minimal medium revealed that the amount of flagellin (FljC), (identified by N-terminal amino acid analysis, AQVINTNSLSL) produced by the slyA mutant was much reduced compared with the parental strain (Fig. 2b). This observation is consistent with the slyA mutant phenotype of reduced survival within macrophages (6) because previous studies have indicated that a flID (encodes a flagellar hook protein) mutant has diminished capacity to survive within macrophages (19, 20). Further investigation revealed altered levels of outer membrane proteins including OmpC, OmpF, PagC (a protein known to be required for full virulence and survival in macrophages), and IroN (a TonB-dependent outer membrane siderophore receptor) when the parent was compared with the slyA mutant (Fig. 2b). In the presence of SlyA the amount of truncated OmpA (M, ~30,000, cf. full-length OmpA 35,000) was greater than in the absence of SlyA, suggesting that SlyA may influence the processing of this protein (Fig. 2b). Mass spectrometry suggested that the ratio of OmpC:OmpF was greater for the parent than for the mutant. Moreover, the slyA mutant possessed reduced amounts of IroN, but the level of PagC was increased (Fig. 2b). These two proteins are both associated with virulence and survival within macrophages, providing a direct link with the slyA phenotype. These data suggest that slyA is important for survival of Salmonella in the stationary phase, consistent with previous observations that slyA expression is enhanced in stationary phase cultures (6), that SlyA can act both as a repressor and activator of transcription, and that the slyA lesion affects the surface and virulence properties of the bacteria.

**Overproduction of SlyA in E. coli and Properties of the Isolated Protein**—To further characterize the SlyA protein it was overproduced in E. coli (JRG4385) as a GST fusion protein. After induction by IPTG (100 μg ml−1 IPTG at 25 °C), the overproduced GST-SlyA fusion protein represented 7% of total bacterial protein and 9% of soluble cell protein. On column cleavage of GST-SlyA with thrombin yielded 9 mg of pure SlyA per liter of culture (Fig. 3). The final product was judged to be 80% pure by SDS-PAGE with two contaminating polypeptides. The major species was shown to be SlyA by N-terminal amino acid sequencing (GSMKLESPLG). The additional N-terminal amino acids, GS, derive from the linker that contains the thrombin cleavage site of pGEX-KG. Recently, it has been suggested that the SlyA protein may initiate not at an ATG codon but at a TTG codon (21), in which case the SlyA protein used here has four additional N-terminal amino acids (GSMK) compared with the native protein. As reported for many other proteins expressed as GST fusions, the two contaminating

**RESULTS**

**Phenotypic Characterization of a slyA Mutant**—It has been reported that slyA mutants are unable to survive within the tissues of the reticuloendothelial system and are hypersensitive to the products of the respiratory burst including hydrogen peroxide (6). Therefore, the interaction of parental and slyA mutant strains of S. typhimurium with J774 macrophages was investigated in a defined Tris-minimal medium. The aerobic growth kinetics of the parent and mutant cultures of the parent and slyA mutant grown in Tris-minimal medium was determined by estimating the number of colony-forming units present by plating serial dilutions on L agar. The error bars indicate the standard deviations from the average of three experiments. b, secreted and outer membrane proteins of aerobic exponential phase cultures of the parent and slyA mutant grown in Tris-minimal medium. SDS-PAGE fractionation of secreted proteins from ST12/75 (lane 1), ST12/75 (lane 2), outer membrane proteins from cultures of ST12/75 (lane 3, ST12/75ΔslyA) (lane 4). The positions of flagellin (FljC), OmpC, OmpA, (and a truncated OmpA, OmpA′), IroN, and PagC are indicated.

**Fig. 2.** Loss of viability of a S. typhimurium slyA strain in Tris-minimal medium. a, the parent (ST12/75, □) and slyA (ST12/75ΔslyA, □) strains were grown in Tris-minimal medium to stationary phase. Samples were taken from the cultures at the indicated times, and the viability of the bacteria was determined by estimating the number of colony-forming units present by plating serial dilutions on L agar. The error bars indicate the standard deviations from the average of three experiments. b, secreted and outer membrane proteins of aerobic exponential phase cultures of the parent and slyA mutant grown in Tris-minimal medium. SDS-PAGE fractionation of secreted proteins from ST12/75 (lane 1), ST12/75 (lane 2), outer membrane proteins from cultures of ST12/75 (lane 3, ST12/75ΔslyA) (lane 4). The positions of flagellin (FljC), OmpC, OmpA, (and a truncated OmpA, OmpA′), IroN, and PagC are indicated.

**Fig. 3.** SDS-PAGE analysis of over-produced SlyA. Lane 1, molecular mass markers (sizes in kDa are indicated); lane 2, crude extract (50 μg); lane 3, SlyA (10 μg).

**Fig. 4.** Interaction of SlyA with the PslyA promoter. SlyA retards the mobility of the SlyA promoter in a band shift assay: lane 1, no protein; lane 2, 0.2 μm; lane 3, 0.4 μm; lane 4, 0.8 μm; lane 5, 1.2 μm; lane 6, 1.6 μm; lane 7, 2.0 μm; lane 8, 3.0 μm; lane 9, 4.0 μm; and lane 10, 5.0 μm. The positions of the free DNA (F) and three SlyA-DNA complexes are indicated.

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polypeptides that co-purified with SlyA were shown by N-terminal amino acid sequencing (GSMKLE) to be prematurely terminated SlyA fragments.

Analysis of the oligomeric state of the purified protein revealed that SlyA is a homodimer, with a $M_r$ of 14,000 by SDS-PAGE, 31,600 by gel filtration, and 33,000 by native polyacrylamide gel electrophoresis. The mass of SlyA as determined by mass spectrometry (16,904 Da) was 57 Da greater than the predicted value of 16,847 Da based on the nucleotide sequence of the cloned slyA gene. The additional mass may represent a posttranslational modification of the SlyA protein, but 57 Da does not correspond to the mass of any of the common protein modifications. The mass discrepancy could be explained by the presence of one iron atom per SlyA monomer, but chemical analysis using bathophenanthroline as an iron chelator indicated that the isolated protein was iron-free. Total metal ion analysis by ICP-MS confirmed the absence of iron ($\leq 0.03$ atoms per monomer) and revealed that no other metals, with the exception of Na$^+$ (70 atoms per monomer) and Ca$^{2+}$ (4.7 atoms per monomer) were present in the isolated protein samples. Thus, it is perhaps more likely that the additional mass is due to retention of one Na$^+$ and one Cl$^-$ (total mass 58.44 Da) adduct from the purification buffer, which contains 150 mM NaCl and 2.5 mM CaCl$_2$. The optical spectrum of the protein was featureless except for an absorbance maximum at 280 nm, indicating the absence of any chromogenic cofactors.

**Interaction of SlyA with the S. typhimurium slyA Promoter Region**—Many transcription factors autoregulate their own expression, and therefore interaction of the SlyA protein with the promoter of the S. typhimurium slyA gene (P$_{slyA}$) was investigated. Bandshift assays with a PCR-generated 424-base pair fragment that extended 287 base pairs upstream of the slyA TTG codon (21) indicated that SlyA could interact with PslyA and that as the concentration of SlyA was increased at least three PslyA-SlyA complexes could be discerned (Fig. 4). The apparent $K_d$ (concentration of SlyA required to retard 50% of PslyA present) was $-0.4 \mu M$.

To test the effects of SlyA on RNA polymerase (RNAP) binding at PslyA, the SlyA protein and RNAP, both individually and in combination, were used in bandshift assays. These experiments revealed that RNAP could recognize PslyA and that adding SlyA did not alter the mobility of the retarded complex (Fig. 5a). When challenged with heparin, a stable PslyA-RNAP complex was observed indicating that RNAP alone is sufficient to generate an open complex at PslyA. However, in the presence of SlyA this heparin-stable complex failed to form and was replaced by a heparin-stable PslyA-SlyA complex (Fig. 5a, lanes 5–8). Thus it would appear that SlyA prevents open complex formation at PslyA and thus negatively regulates its own expression.

To place the SlyA binding sites at PslyA into context it was necessary to attempt to establish the $slyA$ transcription start point. Primer extension analysis with RNA from stationary phase cultures of S. typhimurium suggested that $slyA$ transcription initiated at 41 bases upstream of the translation start point (Fig. 5b). Three bases further upstream there is a potential transcription start point (TTGAGA), thus the $slyA$ transcription start point mapped here is located in an appropriate context.

The position of the transcript start and the inhibitory effects of SlyA on open complex formation at PslyA were confirmed using permanganate footprinting studies, which revealed that T bases at positions $-6$ and $-7$ in the predicted $-10$ element (TATTCT) separated by 17 bases from a potential $-35$ element (TTGAGA), thus the $slyA$ transcription start point mapped here is located in an appropriate context.
within the extended SlyA protected region supported this prediction (Fig. 6b). Combining the five sequences within the SlyA protected region yielded a putative consensus site TTAGCAAGCTAA (Table II).

The role of the heparin-resistant site (site I) in establishing the extended SlyA:DNA complex was investigated by site-directed mutagenesis. Altering this site from TTAGCAAGCTAA to TaCAGAaCTGTA (lowercase indicates a base change), while leaving the other sites unaltered, did not abolish SlyA binding at Polya, but 2-fold higher levels of SlyA were required to begin to retard the mobility of the mutated promoter relative to the unaltered promoter in bandshift assays (Fig. 6c). Footprinting analysis revealed that the SlyA protein still recognized the mutated site (Fig. 6a, lane 7), but that occupation of this region was now abolished by the addition of heparin (Fig. 6a, lane 8). The alterations made to the sequence of site I did not affect the occupation of the other four sites within Polya. These observations are consistent with the reduced affinity of SlyA for the altered promoter and the mobilities of the fully loaded wild type and mutated promoters in bandshift assays (Fig. 6c).

The in vitro data predicted that slyA expression should be negatively autoregulated. Therefore, Polya::lacZ transcriptional fusion was constructed by ligating the same 424-bp EcoRI-HindIII fragment used for the in vitro studies described above into the low copy number lac reporter vector pRWS50 (27), generating the reporter plasmid pGS1384. β-galactosidase activity was measured in parental and slyA mutant strains. After 24 h of aerobic growth at 25 °C the parental (slyA⁺) strain yielded 2034 ± 95 Miller units, whereas the slyA mutant yielded 3332 ± 345 Miller units. Expression of SlyA from its own promoter in multicopy (pGS slightly enhanced the observed repression such that 1627 ± 175 Miller units were recovered. The relatively low level of repression observed might suggest that under the test conditions SlyA is mostly inactive. Alternatively, in vivo only the heparin-resistant SlyA site may be occupied, and from its location relative to RNAP only weak repression would be expected. Nonetheless, the simplest explanation for all of the observations described is that SlyA acts to repress its own expression by promoter occlusion.

Selection of the SlyA Binding Site from a Pool of Random DNA Sequences—The footprinting data described above provided good indications of the sequence of the SlyA binding site, but to better define the site, a SELEX strategy for selecting targets from random DNA sequences was adopted. The approach used was to exploit the GST-SlyA fusion protein by fixing it on small (20–40 µl) GSH-Sepharose columns and passing through samples of DNA containing a 20-bp random sequence, flanked by regions of defined sequence as control columns loaded with GST alone were also used. After three cycles of DNA binding, elution and reamplification by PCR, the GST-SlyA-bound DNA was cloned into pGEM T-easy. The plasmids from 20 individual clones were isolated, and the relevant sections were sequenced. This procedure yielded 20 unique sequences, which were examined for common motifs. The results of the analysis are summarized in Table II, in which the frequencies of a particular nucleotide at each of the 12 positions corresponding to the heparin-resistant site in Polya are provided. Only ligated vector was recovered from the control columns, indicating that the DNA sequences identified from the GST-SlyA columns were specific for SlyA. Thus the putative consensus sequence that emerged from this analysis (t/tg/tg/ a)GCAAGCTAA) was combined with the five sites from Polya to yield a final consensus of TTAGCAAGCTAA.

**DISCUSSION**

The SlyA protein of *S. typhimurium* is a member of a family of transcription factors related to MarR and associated with bacterial adaptation to stress (1, 2, 22). Here it is shown that the SlyA protein is a homodimer that recognizes a partially palindromic DNA sequence consisting of TTAGCAAGCTAA. The dimeric state of SlyA is consistent with recognition of a DNA target consisting of an inverted repeat. In this respect SlyA resembles the MarR protein, which is thought to adopt a dimeric state to recognize an inverted repeat composed of pentameric subelements (23), possibly through interaction with two putative helix-turn-helix motifs (24). Like the Hpr and MarR proteins, SlyA as isolated did not possess, or require, any
co-factors to facilitate binding to target DNA suggesting that the intracellular content of SlyA is regulated and/or that a co-effector can interact with SlyA to abolish DNA binding. The nature of any SlyA co-effector is unknown, but the slyA mutant phenotype suggests that it may be associated with oxidative stress (6) and/or survival in macrophages. The pure SlyA protein now available should allow a range of possible signaling molecules to be tested for modulation of SlyA DNA binding in vitro.

The locations of the SlyA sites within PslyA were consistent with the observed autoregulation of slyA expression observed in vivo. The region occupied by SlyA covers the −10 and −35 promoter elements, thus by binding at these sites SlyA denies RNA polymerase access to the promoter, thereby repressing slyA expression. The size and location of the region of protection and the relatedness of the DNA sequences within the region indicated that at least five SlyA dimers bind at PslyA to repress slyA expression. The multiple PslyA-SlyA complexes observed in bandshifts and the abolition of open complex formation at PslyA in the presence of SlyA supported this conclusion. The latter experiments confirmed the location of the −10 element of PslyA, which had been predicted on the basis of sequence homology, and the position relative to the transcript start. The affinity of SlyA for each individual site within PslyA is likely to be different as indicated by the stepped appearance of the bandshift assays and by the formation of a heparin-resistant PslyA-SlyA complex. Site-directed mutagenesis of the heparin-resistant PslyA-SlyA site indicated that it was not essential for SlyA to bind at PslyA but that the concentration of SlyA required to retard the mobility of PslyA DNA was greater for the altered promoter. This indicates that SlyA binds preferentially at the inverted repeat with a core TTAGC motif that constitutes site I.

The consensus SlyA binding site sequence (TTAGCAAGCTAA) defined here, was used to probe the Salmonella unfinished genomes data base (www.ncbi.nlm.nih.gov/Microbesblast/unfinishedgenome.html) to identify possible members of the SlyA regulon. These searches yielded 17 contigs from S. typhimurium LT2, Salmonella paratyphi, Salmonella enteritidis, and Salmonella dublin, which after further analysis were found to contain SlyA consensus sites upstream of slyA, ispA, and xseB, and encoding proteins with high similarities to OmmpC (ompC) and parainfluenza virus hemagglutinin, respectively. To test these predictions, a Pomp::lacZ fusion was created in pRW50, and β-galactosidase activities were measured for aerobic cultures of parent and slyA mutant strains. The results indicated that omp expression was activated by SlyA (198 ± 11 Miller units for the parental strain and 110 ± 4 Miller units for the slyA mutant). As observed with the PslyA::lacZ fusion, the presence of SlyA had only a small effect on expression suggesting that under the test conditions SlyA is mostly in an inactive state. Nevertheless the simplest interpretation of the data is that the predicted SlyA site located upstream of the ompC coding region is functional in vivo.

The phenotypic tests described here indicated that SlyA is directly or indirectly involved in the regulation fitC, iroN, pagC, and ompC. The available DNA sequences upstream of these coding regions all contained plausible SlyA binding sites (at least 8/12 matches to the consensus) suggesting that SlyA directly regulates these genes. Interestingly, the OmmpC protein has been shown to mediate adherence to macrophages (25) and thus provides a link to the impaired ability of the slyA strain to associate with macrophages (Fig. 1). As it is well established that Salmonella thrives within the intracellular environment, entering macrophages in numbers is a virulence determinant for the bacterium, and from the data presented here it would appear that the slyA mutant is impaired in this respect.

The isolation of the SlyA protein and the definition of its target DNA sequence provide solid foundations for further investigation into the nature of the environmental signals perceived and the network of genes regulated by this important transcription and virulence factor. A variety of strategies have been adopted to obtain a comprehensive picture of Salmonella genes required for intracellular growth and survival, including signature tagged mutagenesis, in vivo expression technology, and selective capture of transcribed sequences, but such approaches rarely identify the same genes, suggesting that several approaches will be needed to obtain such a picture. A useful addition is now provided by definition of the DNA sequence recognized by SlyA, a transcription factor essential for intracellular survival. Upon complete annotation of the Salmonella genome sequence it should be possible to interrogate the data base with the SlyA site for close matches within promoter regions. Such investigations will provide new insight into the mechanisms used by the intracellular pathogen S. typhimurium to evade host defenses and cause disease by unambiguously identifying genes important for survival within macrophages.

**Acknowledgments**—We gratefully acknowledge A. J. G. Moir for DNA and protein sequencing, T. S. Wallis for the lySyl::lacZ fusion, the presence of SlyA had only a small effect on expression suggesting that under the test conditions SlyA is mostly in an inactive state. Nevertheless the simplest interpretation of the data is that the predicted SlyA site located upstream of the ompC coding region is functional in vivo.

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**TABLE II**

Sequences of SlyA binding sites

| Base     | Position |        |        |        |        |        |        |        |        |        |        |
|----------|----------|--------|--------|--------|--------|--------|--------|--------|--------|--------|--------|
| A        | 3        | 7      | 7      | 2      | 2      | 12     | 10     | 1      | 2      | 4      | 12     | 10     |
| T        | 8        | 9      | 2      | 3      | 1      | 6      | 4      | 0      | 12     | 3      | 7      |
| G        | 7        | 1      | 8      | 12     | 4      | 4      | 11     | 6      | 5      | 3      | 1      |
| C        | 3        | 4      | 4      | 14     | 4      | 2      | 5      | 11     | 0      | 3      | 3      |
| SlyA sites |        |        |        |        |        |        |        |        |        |        |        |
| SELEX consensus | T/g | T    | g/a    | A      | A      | G      | C      | T      | A      | A      |
| PslyA I | T        | T      | A      | G      | C      |        |        |        |        |        |        |
| PslyA II| T        | G      | A      | G      | A      | T      | A      | C      | A      | A      |        |
| PslyA III| T      | T      | A      | G      | C      | A      | A      | T      | A      | C      | A      |
| PslyA IV| T        | T      | G      | G      | T      | A      | A      | G      | C      | A      | A      |
| PslyA V | T        | G      | C      | G      | C      | A      | C      | T      | A      | T      | G      | T      |
| PslyA consensus | T    | T      | A      | G      | C      | A      | A      | g/t    | c      | a/t    | A      | A      |
| Combined consensus | T    | T      | A      | G      | C      | A      | A      | G      | C      | T      | A      | A      |
Interaction of SlyA with DNA

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