Abstract

Halsey, Thomas Allen. The Role of SlyA-dependent Genes in *Salmonella* Pathogenesis. (Under the direction of Dr. Stephen J. Libby)

The purpose of this research has been to examine the contribution of the transcriptional regulator, SlyA, to the expression of virulence genes in *Salmonella enterica* serovar Typhimurium. SlyA is a small molecular weight transcriptional regulatory protein that is required for oxidative stress resistance, intramacrophage survival, and for virulence in the murine model of salmonellosis. This work demonstrated that SlyA is required for survival in aerobic environments. A mutation in *slyA* caused profound loss of viability during prolonged stationary phase and *slyA* mutant *Salmonella* grow more slowly as compared to wild type under aerobic conditions. Conversely, under anaerobic conditions, a *slyA* mutant showed a similar phenotype to that of wild type suggesting that a mutation in *slyA* renders it more susceptible to oxidative damage. The transcription of *slyA* was also found to not be significantly affected by other known oxidative stress loci. A transposon insertion into STM2359, however, completely abolished *slyA* expression and this mutant demonstrated many of the phenotypic characteristics of a *slyA* mutant. This work also demonstrates that *slyA* expression can be induced under conditions of low pH and low magnesium ion concentration.

The contribution of the PhoP/Q two-component regulatory system was also examined. As a result of these studies, the *pagC* locus, which was previously thought to be PhoP-dependent, was found to be directly activated by SlyA. Electrophoretic mobility shift analysis and DNase I protection assays showed that SlyA physically interacts with

the *pagC* promoter. Microarray analysis of a *slyA* mutant showed reduced *pagC* expression as compared to wild type. Further analysis by quantitative real time PCR also demonstrated that *pagC* expression is profoundly reduced in a *slyA* mutant *Salmonella*. The greatest reduction in *pagC* expression, however, was illustrated by a mutation in both *slyA* and *phoP*. A *pagC::lacZ* promoter fusion combined with a mutation in *slyA, phoP*, or both also confirmed these observations. These studies also demonstrated that PhoP contributes to *pagC* expression indirectly by possibly influencing the specificity of SlyA.

The work presented here also illustrated a role of SlyA in the activation of *Salmonella* pathogenicity island 2 gene expression. These studies demonstrated that SlyA binds to the *ssrB* promoter by electrophoretic mobility shift analysis and DNase I protection assays. Furthermore, these studies showed that SlyA directly induces *ssrB* expression as determined by microarray analysis, quantitative real time PCR, and promoter fusions. The competitive infection data also suggests that SPI2 gene expression and SlyA are part of the same pathway. Collectively, these data show a direct connection between SPI2 and SlyA.

The Role of SlyA-dependent Genes in *Salmonella* Pathogenesis

by
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A dissertation submitted to the Graduate Faculty of
North Caroline State University
in partial fulfillment of the
requirements for the Degree of
Doctorate of Philosophy

MICROBIOLOGY

Raleigh

2003

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DEDICATION

I dedicate this work to the two most important women in my life: my wife, Joy, and my daughter, Madison, and also to my family. Without their love and support, this work would not have been possible.

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ACKNOWLEDGEMENTS

I would like to thank my wife and daughter and also my parents for all of their love and support they have given me over the years. I would also like to thank my brother and sister for their love and positive encouragement. I give a special acknowledgement to my mentor, Dr. Stephen J. Libby, for his support in putting up with me over the years, his unwavering confidence in my abilities as a scientist, and for always having an open door. I would also like to thank Gracie Thomas, Beth Boutt, Chris Allen, Jennifer Potter, and all the undergraduates in the lab over the years for keeping things interesting. I offer my gratitude to the other members of my committee, Dr. Geraldine Luginbuhl, Dr. Hosni Hassan, Dr. Craig Altier, and Dr. Paul Orndorff. Their input and guidance has been very important in making this work possible.

TABLE OF CONTENTS

LIST OF TABLES

THE ROLE OF *SLYA* IN OXIDATIVE STRESS RESISTANCE IN *SALMONELLA ENTERICA* SEROVAR TYPHIMURIUM

UNRAVELING THE SLYA AND PHOP REGULONS IN *SALMONELLA ENTERICA* SEROVAR TYPHIMURIUM

THE TRANSCRIPTIONAL REGULATOR, SLYA, IS REQUIRED FOR SPI2 GENE EXPRESSION IN *SALMONELLA ENTERICA* SEROVAR TYPHIMURIUM

LIST OF FIGURES

THE FERRITIN-LIKE DPS PROTEIN IS REQUIRED FOR *SALMONELLA ENTERICA* SEROVAR TYPHIMURIUM OXIDATIVE STRESS RESISTANCE AND VIRULENCE

THE ROLE OF *SLYA* IN OXIDATIVE STRESS RESISTANCE IN *SALMONELLA ENTERICA* SEROVAR TYPHIMURIUM

UNRAVELING THE PHOP AND SLYA REGULONS IN *SALMONELLA ENTERICA* SEROVAR TYPHIMURIUM

THE TRANSCRIPTIONAL REGULATOR, SLYA, IS REQUIRED FOR SPI2 GENE EXPRESSION IN *SALMONELLA ENTERICA* SEROVAR TYPHIMURIUM

CHAPTER 1

Review of *Salmonella* Pathogenesis

Introduction to the Genus

The first strain of *Salmonella* was isolated from the intestine of a pig by the American veterinary scientist Daniel E. Salmon in 1885. This strain was called *Salmonella choleraesuis*. The genus *Salmonella* encompasses a very large, but genetically related taxonomic group. At present, there are over 2,463 recognized serotypes of *Salmonella* (150). This classification is based on the Kauffman-White scheme of classification and is based on the serological characteristics of the H-flagellar antigen and the O-somatic antigen (21, 109). The complete formulae, characteristics, new serotypes, and a complete registry of the *Salmonella* genus are maintained by the WHO Collaborating Centre for Reference and Research on *Salmonella* at the Pasteur Institute, Paris, France (149, 150). *Salmonella* nomenclature has been complex, but with the use of DNA-DNA hybridization studies, multilocus enzyme analysis, and genome sequence projects, the genus has been subdivided into five groups. In 1986, the International Congress of Microbiology recommended that the type species of *Salmonella* be changed to *S. enterica* (144). The previous individual serotype names would still be used, but would not be in italics, i.e. *Salmonella enterica* serovar Typhimurium would be used in place of *Salmonella typhimurium*. In 2000, the American Society for Microbiology adapted this nomenclature with rules for defining a particular strain: *Salmonella enterica* serovar Typhimurium for the first usage, then *Salmonella* serovar Typhimurium in subsequent references to the strain in the text of an article. This taxonomically correct method, but longer method of describing a particular strain of

Salmonella is slowly being adopted in the current literature. For the rest of this thesis, I will use the *Salmonella* Typhimurium nomenclature, shortened to *S.* Typhimurium.

General Characteristics

Salmonella are Gram-negative facultative bacilli that are able to carry out both respiratory and fermentative metabolism. *Salmonella* grows optimally at 37^0C and all but two serovars, *Salmonella* serovar Pullorum and Gallinarum, are motile with long, peritrichous flagella. With the exception of *Salmonella* serovar Arizonae and Diarizonae, *Salmonella* are non-lactose fermenting. Generally, *Salmonella* are able to catabolize glucose and other carbohydrates to produce acid and gas and produce hydrogen sulfide (H_2S) with exception of *Salmonella* serovar Typhi and Paratyphi. *Salmonella* are oxidase positive, indole and Voges-Proskauer negative, methyl red and Simmons citrate positive, lysine and ornithine decarboxylase positive, and are able to utilize a variety of sugars as a carbon source. Common biochemical assays utilized for the presumptive identification of *Salmonella* are growth characteristics in TSI tube (triple sugar iron agar). *Salmonella* are indole negative when tested with Kovac's reagent with no reported exceptions. *Salmonella* are profoundly resistant to bile salts and this property is the basis for their growth on MacConkey, Salmonella-Shigella agar, XLT and XLD agar, and Hektoen enteric agars. Regardless of serovar, most *Salmonella* are catalase positive.

Disease caused by *Salmonella*

Human infections with both typhoidal and non-typhoidal *Salmonella* continue to pose a major health problem worldwide. There are an estimated 40,000 cases of salmonellosis

reported each year in the United States, but a low reporting rate of 1 to 5% translates into an estimated 2-4 million cases per year (129). Some species of *Salmonella* have a narrow host range, such as *S.* Typhi in humans, however, *S.* Typhimurium has a broad host range including humans, domestic animals, and wildlife. Infection with *Salmonella* in humans usually results from the ingestion of contaminated food and water, where symptoms of disease range from a self-limiting gastroenteritis to a more life-threatening acute systemic salmonellosis. Symptoms of *S.* Typhi infection in humans include high fever (103-104^{o}F), stomach pains, loss of appetite, and fatigue. If left untreated, as many as 20% of persons infected with *S.* Typhi may die from complications of the disease, however, a full recovery can be expected with prescribed antibiotics. In addition, some persons infected with *S.* Typhi can become carriers of the disease and spread it to other people through food handling. *S.* Typhimurium infection in mice causes systemic disease that closely parallels that of human typhoid or enteric fever caused by *S. typhi* infection in humans. Infection of mice with *S.* Typhimurium is now the model system to study human infection with *S.* typhi.

Mice are an excellent model system in which to study human salmonellosis because the immune systems of mice and humans are quite similar and genetic tools are available to study the mechanisms of infection and immunity. Genetic studies in the mouse enabled researchers to map mutations on mouse chromosome 1 that conferred resistance to three intracellular pathogens (*Mycobacterium bovis* BCG*, Salmonella typhimurium,* and *Leishmania donovani*) (20, 146, 147, 157). Subsequently, researchers determined that the mutations were in a single gene at this chromosomal location now designated *Bcg/Ity/Lsh* (20, 147, 157). *In vivo* and *in vitro* studies have since demonstrated that this locus is important in the initial phase of infection, primarily by controlling the intracellular microbial

replication (81). The gene encodes a 90-100 kDa integral membrane phosphoglycoprotein called the natural resistance-associated macrophage protein 1 (*Nramp1*). This protein is expressed in the phagosome of macrophages and is responsible for the transport of divalent metal cations against a proton gradient and macrophage activation (15, 63). The mutation that makes mice susceptible to *Salmonella* infection lies in a glycin-to-aspartate substitution in the TM4 domain of the Nramp1 protein (75, 178).

Toll-like receptors are also an important part of host innate immunity to microbial infection. Many of these receptors have evolved to recognize unique products of microbial metabolism, such as lipopolysaccharide. Mouse Toll-like receptor-4 has been identified as a signal transducing receptor for LPS and *Tlr4* knockout mice show increase sensitivity to LPS (148, 151). Studies of Sultzer demonstrated that inbred C3H/HeJ mice were innately resistant to lethal LPS challenge and their macrophages hyporesponsive to LPS *in vitro* (166). Genetic analysis has revealed that this phenotype is due to the LPS responsive allele (*Lps*) which can come in two forms: the normal allele, Lps^n and the hyporesponsive allele, Lps^d (179). The hyporesponsive allele has also been found to be associated with a reduction in LPS-mediated macrophage activation and survival of infection with *S*. Typhimurium (125). C3H/HeJ mice are profoundly susceptible to *S.* Typhimurium infection due to a point mutation in Tlr4 which prevents downstream signaling (130, 148, 151). The recent advances in mouse genetics and the availability of transgenic mice has enabled researchers to examine many of the *Salmonella*-host interactions and better understand the aspects of *Salmonella* pathogenesis.

Disease Mechanisms of *Salmonella*

In order to cause systemic disease in its host, pathogenic *Salmonella* spp. must be able to withstand gastric acidity, invade the intestinal epithelium, and survive and replicate within host phagocytes. *Salmonella* also must be able to adhere to and invade the epithelial cell layer lining the intestine in order to cause enteritis (170). The primary site of invasion is initiated at Peyer's patches in specialized cells called M-cells (99, 103, 104). M-cells are specialized epithelial cells that are responsible for antigenic sampling of the lumen contents and presenting it to the immune system. *Salmonella* interaction with these M cells facilitates host colonization during macropinocytosis through a mechanism of cell membrane ruffling which results in the internalization of the bacteria into membrane-bound endocytic vacuoles (60, 65, 71, 105, 128, 167). Once the bacterium has transcytosed the intestinal epithelium, it enters the follicle dome of the lamina propria and encounters host phagocytes. Most *Salmonella* have the ability to survive and replicate within these cells which leads to the extra-intestinal colonization of other organs of the body including the spleen and liver (56).

Salmonella diverged from its closest relative, *E.coli*, approximately 100 million years ago (140). Evidence for this divergence is found in the identification of large clusters of conserved virulence genes located in chromosomal sequence of pathogenic *Salmonella* spp. that are absent from the analogous region of non-pathogenic spp. such as *E. coli* K12. These regions of chromosomal DNA are referred to as a *Salmonella* Pathogenicity Island (SPI) (19). The GC content of these islands is much lower than that of the remaining chromosomal DNA sequence, suggesting that the pathogenicity islands were attained through a horizontal transfer event from a phage or plasmid from an unknown source. Interestingly, the insertion sites of many of these pathogenicity islands are near tRNA genes. These tRNA genes are

highly conserved among bacteria and are known to serve as anchor points for temperate phages (100). All of the *Salmonella* pathogenicity islands, with the exception of SPI-1, have been found to be associated with tRNA genes (16, 95, 181, 182). Specifically, the SPI-2 locus has been found to be associated with the $tRNA^{ValV}$ gene (95), the SPI-3 locus has been found to be associated with the $tRNA^{SelC}$ gene (16), the SPI-4 locus has been associated with a putative tRNA gene sequence (181), and SPI-5 has been found to be associated with the $tRNA^{SerX}$ gene (182). Other bacterial pathogens such as *E.coli*, *Shigella*, *Vibrio cholera*, *Pseudomonas syringae*, and *Helicobacter pylori* possess pathogenicity islands as well (19, 28, 44, 102, 106, 152). Thus far, five pathogenicity islands have been identified in *S.* Typhimurium and *S.* Typhi (78, 181, 182).

Salmonella Pathogenicity Island 1

Salmonella induces its uptake by the intestinal epithelial cells through the action of a Type III secretion system that has been designated SPI1 (68, 133). This island comprises a 40-kilobase region of DNA that encodes a secretion apparatus, transcriptional regulators, and secreted effector proteins. SPI1 is found in all *Salmonella* and is located at centisome 63 on the *Salmonella* chromosome map and contains the *inv/spa* cluster. There are over 29 genes encoded within SPI1 that are involved in the assembly of a type III secretion apparatus vital for entry into host epithelial cells (34, 35). This type III secretion system spans both the inner and outer membrane and resembles a slender, needle structure that protrudes out from the outer membrane (113). The protein components of the base and needle structure have been identified and include: PrgH, PrgK, and InvG, which make up the base structure, and the major component of the needle structure, PrgI (114).

Environmental stimuli similar to those found in the intestinal tract are know to regulate SPI1 invasion genes (65, 69, 116). Regulation of SPI1 is also controlled by several proteins, including HilA and InvF, which are encoded within SPI1 itself, and others that are encoded in regions outside SPI1 (5, 9, 135). HilA is a transcriptional regulator whose expression is controlled by HilC and HilD (121, 155). InvF regulates the expression of secreted SPI-1 effector proteins both dependently and independently of *hilA* (45, 122). Recent studies have also shown that invasion genes are repressed by PhoP/PhoQ, however, they are positively regulated by BarA/SirA (1, 6, 14, 143). Recent studies have also determined that invasion gene expression is regulated by CsrA/CsrB (5).

The effector proteins encoded within SPI1 are translocated into the host cell cytoplasm through the secretion apparatus. In particular, AvrA, SipABCD, SopE, SopE2, SopB, and SopD are translocated by the secretion machinery encoded within SPI1. Specifically, SipA is secreted into the host enterocyte and binds to the actin cytoskeleton preventing actin rearrangements and inducing membrane ruffling (187). SipB activates caspase-1 which has been found to induce apoptosis within epithelial cells (98). Studies have also shown that SopE and SopE2 are responsible for activating Rho GTPases including cdc42 and Rac, which influence cytoskeletal rearrangements (10, 91, 165). SopB encodes an inositol phosphate phosphatase that induces secretory diarrhea by blocking chloride channel closure (137). In the murine model of salmonellosis, SPI1 mutant *Salmonella* are avirulent by the oral route of infection, however, virulence is restored when given intraperitoneally or intravenously indicating that SPI1 is required for invasion, however, it is not required for systemic infection (134).

Salmonella Pathogenicity Island 2

Studies have shown that another pathogenicity island, SPI2, is essential for intramacrophage survival (32, 96, 139). The SPI2 gene cluster is located at centisome 30.7 on the *Salmonella* chromosome and comprises 40 kilobases that contains 44 open reading frames, many of which encode the two component regulatory system SsrAB and a type III secretion apparatus. Interestingly, functional analysis has reveled that only a 25 kilobase portion of the 40 kilobase insertion is required for virulence, which encodes SsrAB and the type III secretion apparatus (90). SPI2 mutants are profoundly attenuated for virulence whether administered orally or intraperitoneally and cannot proliferate in the spleen or liver of infected mice suggesting that SPI-2 is required for the systemic phase of infection (156).

In vitro, SPI2 gene expression is induced in response to low levels of magnesium or calcium or by phosphate starvation (32, 42, 174). Regulators of SPI2 include SsrA/SsrB, EnvZ/OmpR, which regulates SsrA/SsrB and presumably responds to low osmolarity in the phagosome (32, 40, 73, 115). Elegant studies by Kenney *et* al. (52) have revealed that phospho-OmpR activates the SsrA/B locus by direct interaction at their respective promoters. The predicted structure of SsrA is that of a phosho-relay type sensor kinase similar to BarA or ArcA while SsrB has homology to UvrY of *E. coli* and SirA of *Salmonella* (41). Recent studies by Hensel, Cirillo, and Vazquez-Torres have demonstrated that the SPI2 genes are required for survival in host phagocytes (33, 97). Subsequent research by Vazquez-Torres *et al*. suggests that SPI2 may interfere with the trafficking of the NADPH oxidase to *Salmonella*-containing vacuoles thereby preventing phagocyte-dependent oxidative killing (177). There was a 10-fold reduction of co-localized phagosome-associated NADPH oxidase in wild type *Salmonella*-infected macrophages as compared to a SPI2 mutant (177). This

suggests that the secreted protein products from the SPI2 locus deflect trafficking of the vesicles containing NADPH oxidase to *Salmonella*-containing phagosomes thereby preventing *Salmonella* exposure to toxic reactive oxygen species. An explanation of this observation may relate to the SpiC protein, encoded within SPI2 that is secreted into the macrophage cytosol (172). A functional SpiC protein is required for the prevention of endosomal or lysosomal fusion to *Salmonella*-containing phagosomes (176).

Salmonella Pathogenicity Island 3

Salmonella spp. also encode a third pathogenicity island designated SPI3. SPI3 is a 17 kb region located at centisome 82 on the *Salmonella* chromosome and encodes the *mgtCB* operon that is required for both growth in Mg^{+2}-limiting conditions and intramacrophage survival (17, 158). This region also encodes four more genes, *rmbA, misL, fidL,* and *mart*, which show similarity to genes encoded within *E.coli* K-12, however the genetic organization is different (18, 36). These genes encode proteins that include putative cytoplasmic proteins and putative membrane proteins. The two-component system, PhoP/PhoQ, regulates this operon through the Mg^{+2} sensor, PhoQ, and the activator, PhoP (73, 161). SPI3 has been shown to be required for survival in macrophages and virulence in the murine model of salmonellosis (16).

Salmonella Pathogenicity Island 4

Salmonella pathogenicity island 4, SPI4, was originally described as a 25 kb pathogenicity island that encoded 18 open reading frames designated A-R (181). The recently completed annotation of SPI-4, however, has revealed that SPI-4 actually encodes 6

open reading frames with SM4261 encoding a 660 kDa protein (127). SPI4 gene expression is regulated by the transcriptional regulator, SirA, and SPI4 is thought to play a role in invasion (1, 3). Allen *et* al. was the first to show that the genes encoded within SPI4 play a role in the invasion of cultured epithelial cells (3). This finding is significant because Murray *et* al. Showed that when SPI1 is completely deleted, the ability of *Salmonella* to invade was not completely eliminated (135). Unpublished data of Libby *et al.*, however, suggests that SPI4 may be required for intramacrophage survival and virulence in mice when infected intraperitoneally.

Salmonella Pathogenicity Island 5

Salmonella Pathogenicity Island5 was first identified in *S. Dublin* at centisome 25 on the chromosome (135, 182). Like SPI-4, SPI5 is only found in *Salmonella* spp. and is absent from other enteropathogenic bacteria such as EPEC, *Yersinia pseudotuberculosis,* and *Shigella sonnei* (182). The five genes encoded within SPI5 include: *pipA, pipB, pipC, pipD,* and *orfX*. A mutation in any one of these genes results in diminished fluid/chloride secretion and poor inflammatory responses when assayed in the bovine ileal loop model (182). Also encoded within SPI5 is *sopB* that codes for a protein required for fluid secretion and neutrophil recruitment, however the protein is actually translocated by the SPI1 *inv*/*spa* locus (67, 72, 92, 182). Collectively, these data demonstrate that SPI5 is required for enteric, but not systemic salmonellosis. This is a clear example of the intricate and complicated mechanism of cross-talk between genes located on different pathogenicity islands.

The *Salmonella* Virulence Plasmid

Most *Salmonella* strains harbor virulence plasmids that encode genes required for the ability to cause systemic disease with the exception of *S*. Typhi (85). Isolates of *S*. Typhimurium carry a 90 kb virulence plasmid with a conserved 8 kb region encoding the *spv* genes (66, 83). The *spv* locus encodes five genes that contribute to the interaction of *Salmonella* with host cells and these genes also affect bacterial growth during the systemic phase of infection inside of macrophages (86). Recent studies have demonstrated that SpvA is a negative regulator of the *spv* operon, while SpvD is most likely secreted (46). It has also been shown that SpvB encodes an ADP-ribosylating enzyme that is essential for virulence in the murine model (118). Expression of *spvABCD* relies on both SpvR positive activator and RpoS (84). The virulence plasmid also encodes additional proteins that are important for *Salmonella* pathogenesis. The plasmid-encoded fimbriae region, *pef*, is involved in the attachment to the intestinal epithelium (12). Rck is an outermembrane protein that is involved in intestinal epithelial cell invasion and resistance to host complement factors and this protein is also encoded within the virulence plasmid, but is only found in *S*. Typhimurium (31).

Survival in the Phagocyte: The Role of the Respiratory Burst

Salmonella species are pathogens that have the unique ability to survive within the phagosomal compartment of macrophages. Survival within this compartment requires that *Salmonella* be able to withstand both the oxygen-dependent and oxygen-independent killing mechanisms of professional phagocytes. The oxygen-independent mechanisms of killing by host phagocytes include reduced phagosomal pH, nutrient limitation, and antimicrobial

peptides (58). The oxygen-dependent killing mechanisms of professional phagocytes are the focus of this review and will be discussed in detail below.

Oxidative killing by professional phagocytes is oxygen-dependent and involves the production of toxic and highly reactive molecules that can damage microbial DNA, protein, and lipids (43). As a result of this selective pressure, many bacteria have evolved oxidative stress resistance mechanisms that are essential for their survival within the phagosomal compartment. The ability of *Salmonella* to survive and replicate within host macrophages is an essential aspect of its ability to cause systemic infection (53, 54, 57-59). Intense effort has been directed at understanding how *Salmonella* is able to survive and replicate within host macrophages and these studies have yielded several genes that are required for intramacrophage survival and virulence. These genes include: *slyA, phoP,* SPI2*, mig-14, recA, rpoE, rpoS,* and *pagC*, among many others (24, 29, 30, 38, 49, 94). Oxidative stress resistance in *E. coli* and *S.* Typhimurium has been shown to involve more than 60 genes (50). Interestingly, many of the genes that are required for oxidative stress resistance are not required for intramacrophage survival and virulence. For example, a *Salmonella* strain harboring mutations in two catalase genes required for hydrogen peroxide resistance, *katE* and *katG*, is sensitive to *in vitro* derived oxidative stress, however, it remains fully virulent in the murine model of salmonellosis (24). Of the genetic loci that are required for intramacrophage survival and virulence, only *slyA*, *sodC*, *rpoE*, *recA*, and *recBC* have clearly been demonstrated to be required for oxidative stress resistance *in vitro* and they are also essential for intramacrophage survival and virulence in mice *in vivo* (11, 23-25, 168).

The respiratory burst of professional phagocytic cells, including neutrophils, involves the production of superoxide anion (O_2^-) from the univalent reduction of molecular oxygen

through the action of NADPH oxidase (8). Specifically, upon phagocytosis of *Salmonella*, the p67, p47, and p40 cytosolic components of the NADPH oxidase translocate via the cytoskeleton for association with p22, gp91, and Rap1A membrane-bound subunits along with the cytosolic Rac proteins to the *Salmonella*-containing phagosome (177). As a result, superoxide anion is produced that can dismute to form hydrogen peroxide (H_2O_2). Unlike superoxide, hydrogen peroxide is a membrane-permeable species that is capable of reacting with lipids, proteins, and DNA (176). Hydrogen peroxide can also react with Fe (II) in the Fenton reaction to produce the toxic hydroxyl radical (HO•). Superoxide can also react with nitric oxide (NO•), which leads to the production of peroxynitrite ($ONOO^-$). Hydrogen peroxide can also react with NO• by a mechanism that appears to be iron-dependent (142). Without microbial mechanisms of resistance to these compounds, survival within the phagosome is virtually impossible.

Studies aimed at understanding how *Salmonella* is able to survive within such as hostile environment has led to the discovery of several genetic and biochemical factors that are used for *Salmonella* survival. The importance of the respiratory burst in resistance to *Salmonella* has been shown by the observation that *Salmonella* mutants defective in DNA repair genes, *recA* and *recBC*, are able to survive and replicate within murine macrophages lacking the ability to generate a respiratory burst, however, they are killed in burst-competent macrophages (24). Mice that have a defined mutation in the gp91 subunit of the NADPH oxidase (*phox* knockouts) are susceptible to *Salmonella* infection and macrophages from these mice are unable to control *Salmonella* replication (38). A *Salmonella* mutant lacking *sodA*, the Mn-cytoplasmic superoxide dismutase, remains virulent in these mice and is able to replicate in macrophages. Recent studies have demonstrated that *Salmonella* contains two

periplasmic Cu, Zn-SODs, *sodCI* and *sodCII*, that are important for protection against exogenous oxidative damage (48). The first gene to be discovered is now designated *sodCI* which is encoded on a cryptic λ–like bacteriophage (38, 51), while the second gene is now designated *sodCII* and is more closely related to the *E.coli sodC* gene (48). The sodCII gene is maximally expressed in stationary phase in an RpoS-dependent manner while sodCI seems to maximally expressed at the transition between exponential and stationary phase independent of RpoS (48, 49, 74, 117). Mouse virulence studies have demonstrated that a SodC mutant is attenuated for virulence in mice and are unable to replicate in respiratory-competent macrophages, however, restoration of virulence and macrophage growth can be observed in gp91*phox*$^{-/-}$ mice (38, 171).

The Non-specific DNA-binding protein Dps

In *S.* Typhimurium, activation of specific regulons responsible for oxidative stress resistance are equally important for survival, whether in the environment or within the host. The accumulation of reactive oxygen species is inevitable; in aerobic environments, within host phagocytes, and during periods of stationary phase survival. As a result of increased oxidative stress during stationary phase, a number of stationary phase-specific genes are expressed for the resistance to these stresses. Of particular importance is the stationary phase-specific sigma factor σ^S, which is required for oxidative stress resistance during stationary phase (49). A number of genes are regulated by *rpoS* during stationary phase including the DNA-binding protein in stationary phase, Dps, that is required for starvation-induced resistance to hydrogen peroxide (4). Dps is a low molecular weight protein accumulates during stationary phase and binds to DNA (4). As *E.coli* enters stationary

phase, more than 180,000 Dps molecules accumulate within a single organism, making Dps the most abundant protein within the cell (2). Expression of *dps* in *E.coli* has been shown to be regulated by RpoS (σ^{38}), OxyR, and IHF (7). Dps is able to co-crystallize with bacterial DNA and this is thought to protect the nucleic acid from damage during conditions of oxidative stress (180). The exact mechanisms of protection have been the focus of intense study in recent years.

The exact role of Dps in oxidative stress resistance is thought to be that of direct DNA protection, however, there has been some debate as to this finding. It is known that Dps is required for starvation-induced H_2O_2 resistance in *Escherichia coli* (4). Expression of Dps has also been shown to be critical for survival during oxidative stress, regardless of whether or not the cells are actively growing or not (7, 120). Dps mutant *Escherichia coli* are very sensitive to H_2O_2 in stationary phase and also show an irregular pattern of protein synthesis (126). Zheng *et* al. (186) have also recently shown that *dps* is the most strongly hydrogen peroxide-induced gene using DNA microarray-mediated transcriptional profiling. In addition, studies by Valdivia *et* al. (173) have demonstrated that Dps is one of the most abundant proteins expressed within the intracellular environment of macrophages.

The regulation between the assimilation and storage of iron is believed to prevent free iron accumulation, protecting the cells against iron toxicity (93, 111). Dps may function to block the association of Fe (II) with hydrogen peroxide in the Fenton reaction, which leads to the production of the highly reactive and toxic hydroxyl radical. Recently, Zhao et al. (185) have found that *E. coli* Dps may provide defense against Fe(II) and reactive oxygen species through its spatial association with DNA and it capacity to bind Fe(II) and consume hydrogen peroxide without the production of hydroxyl radical. Dps homologs have also been

found in a wide and diverse range of bacteria, including *Campylobacter jejuni* (101). Recent studies have demonstrated that the Dps protein of *C. jejuni* contributes to protection against oxidative stresses by sequestering cellular free iron to prevent generation of hydroxyl radicals under hydrogen peroxide stress via the Fenton reaction (101). This finding is in direct contrast with the finding that Dps functions to bind bacterial DNA and protect it from oxidative stress. Recent studies in *Agrobacterium tumefaciens* and *Mycobacterium smegmatis* indicate that Dps can prevent oxidative DNA damage even in the absence of DNA binding (27, 89). The conservation of Dps and the recent reports that the protein is necessary for the avoidance of oxidative stress generated via the Fenton reaction suggest that Dps may represent a conserved non-enzymatic mechanism of protection against oxidative stress. Furthermore, this protein may function differently depending on the primary environment that the microorganism is exposed to within its niche. Taken together these data suggest that Dps is an evolutionarily conserved protein that is essential for oxidative stress resistance across species.

The PhoP/PhoQ Two-component System

A model two-component regulatory system consists of a sensor histidine kinase and response regulator, which includes a receiver domain and a DNA-binding domain. PhoP/PhoQ is a two-component regulatory system that is required for virulence, is responsible for the adaptation to Mg^{2+}-limiting environments, and regulates numerous cellular activities in many Gram-positive bacteria, including *Mycobcterium tuberculosis*, and Gram-negative bacteria, including *Salmonella*. This system is encoded by the *phoP* locus, which was first identified in *Salmonella* as controlling the expression of a nonspecific acid

phosphatase (110). The PhoP/PhoQ system has best been described and characterized in *Salmonella*, however, homologues in other microorganisms demonstrate similar findings to those described in *Salmonella*. The PhoP-PhoQ homologue in *M. tuberculosis*, PhoP-PhoR, is required for intracellular growth within macrophages and virulence in mice (145). A PhoP-PhoQ homolog has been identified in *Pseudomonas aeruginosa* where it is required for resistance to antimicrobial peptides and is regulated by Mg^{2+} (47, 124). A homolog has also been identified in *Erwinia carotovora* (PehR-PehS) that responds to Ca^{2+} and regulates the virulence protein PehA (61, 62).

PhoQ is the protein of this system that responds to these environmental stimuli presumably through its periplasmic domain, which contains several acidic amino acid residues. The protein also features two transmembrane regions that make up a long cytoplasmic tail that contains a histidine residue predicted to be the site of autophosphorylation. High Mg^{2+} promotes the dephosphorylation of phospho-PhoP by the PhoQ protein in a mechanism in which a phosphate group is transferred from aspartate 57 on PhoP to histidine 247 on PhoQ (26). Although this system is an example of a model two-component system, it is the first example of a regulatory system that responds to extracellular Mg^{2+} as a primary signal. Several studies have demonstrated that growth in micromolar concentrations of Mg^{2+} promotes the transcription of PhoP-dependent genes, however, growth in millimolar concentrations of Mg^{2+} represses the expression of these genes to levels seen in *phoP* or *phoQ* null mutants (73, 108, 161). In addition to Mg^{2+}, Ca^{2+} and Mn^{2+} can repress transcription of PhoP-regulated genes, however, other divalent cations such as Ni^{2+} and Cu^{2+} show no effect (73)

The understanding of PhoP-dependent gene expression has been the focus of intense studies in recent years. A direct repeat, (T/G) GTTA has been identified in the promoter regions of the *Salmonella phoPQ* (162) and *phoN* (80) genes, however, it has not been found in many other genes that are thought to be PhoP regulated. There are several genes that have been identified as PhoP-dependent, however, their function is largely unknown. These genes, designated PAGs for PhoP-activated gene, and PRGs, for PhoP-repressed genes, are thought to play a role in *Salmonella* pathogenesis, however, the exact role has yet to be elucidated. Many of these genes encode putative membrane proteins that are thought to play a role in antimicrobial peptide resistance, however, this suggestion has not been supported.

It is known that the PhoP-PhoQ system controls the expression of several genes that are required for growth in low Mg^{2+} concentrations (160). These include the *mgtA* and *mgtBC* genes, which encode a magnesium transport system and are encoded within SPI3 (159). Null mutants of any one of these genes show impaired growth in low magnesium and these mutants also show the loss of viability when grown on solid media (160). The PhoP-PhoQ system also regulates another subset of genes that are required for growth in magnesium-limiting conditions. The *ugd* and *pbgPE* –encoded proteins have been shown to not only be required for growth in low magnesium concentrations (160), but they are also required for LPS modifications that are required for *Salmonella* resistance to the antimicrobial peptide polymyxin B (77, 87). Resistance to other antimicrobial peptides, bile salts, and acid pH may also be dependent on PhoP-PhoQ as *phoP/phoQ* null mutants show increased susceptibility to these conditions (13, 53, 64, 79, 82, 88, 132, 175). Interestingly, another two-component system, the PmrA-PmrB system can also activate the loci responsible for antimicrobial peptide resistance. Activation of this system has been found to occur in the

presence of high Fe^{3+} (183), however, this system can also occur in magnesium-limiting conditions through the *pmrD* gene, which itself is regulated by PhoP (107, 112). Another gene that has been identified as PhoP-dependent is *mig-14*, which has been found to be required for resistance to polymyxin B (22).

The PhoP-PhoQ system is unequivocally required for intramacrophage survival and virulence in the murine model of salmonellosis (55, 76, 131). Null mutants of either *phoP* or *phoQ* are profoundly attenuated in BALB/c mice with median lethal doses that are 5 orders of magnitude higher than that of wild type *Salmonella* (53, 70, 131). The attenuation that is seen in these mutants may be due to their inability to survive in macrophages. Several PhoP-dependent genes have been shown to be required for survival in macrophages and the *mgtC* gene appears to be the gene primarily responsible for this phenotype. MgtC is required for both survival in macrophages and virulence in mice through its ability to control proper Mg^{2+} concentrations within the phagosomal compartment of macrophages (16). In addition, the intramacrophage survival defect of both *phoP* and *mgtC* null mutants can partially be restored by the addition of Mg^{2+} to the tissue culture medium (16). Although MgtC is important for intramacrophage survival and virulence in mice, *phoP* null mutants are considerably more attenuated than *mgtC* mutants. This data indicates that there are perhaps more PhoP-dependent genes that are required for *Salmonella* virulence. The identification of other PhoP-dependent loci remains the focus of intense study and the unraveling of the PhoP regulon will be instrumental in understanding how PhoP functions to regulate the systemic phase of *Salmonella* infection.

The Role of SlyA in *Salmonella* Pathogenesis

The *slyA* gene was initially identified in association with hemolytic activity in *S.* Typhimurium ATCC 14028s (119). The hemolytic activity was initially ascribed to SlyA itself, however, subsequent studies revealed that SlyA was responsible for the expression of a cryptic pore-forming hemolysin encoded by *clyA* or *sheA* in *E. coli* (123, 141). Subsequent research has now demonstrated that SlyA is a transcriptional regulatory protein that is required for the expression of several different genes, many of which have yet to be identified (39, 123, 163). SlyA is a member of the MarR family of transcriptional regulators that are found in a diverse group of both pathogenic and non-pathogenic bacteria. The pathogens include: *M. tuberculosis, E. chrysanthemi, C. diversus, S. flexneri, Y. enterocolitica, S. marcesens,* and *P. carotovora* (169). Of the bacterial and archeal genomes that have sequenced completely or are in process, 90 of 146 (62%) harbor a *slyA* sequence, and 84 of 146 (57%) have a *marR* gene, representing 60 genera. Although SlyA is related to MarR in amino acid sequence with a similarity of 48%, and there are conserved regions of sequence, a recent study has demonstrated functional differences. MarR is a repressor protein that is capable of binding only at one portion of the *mar* operon, *marO*. The crystal structure of SlyA from *Entercoccus faecalis* has recently been determined and it demonstrates that MarR and SlyA show local divergence in sequence and structure that allows them to respond to different signal molecules and to bind to diverse DNA targets by using alternative modes of action (184).

The role of *slyA* in the pathogenesis of a variety of bacterial pathogens has been the focus of intense study in recent years. Many independent studies have found that *slyA* is crucial for virulence in a variety of pathogens. Inactivation of the *slyA* homolog in *E.*

chrysanthemi abolishes expression of several important virulence factors needed for colonization and development of disease (154, 169). The *hor* gene from *P. carotovora* controls antibiotic and exoenzyme production and mutants are significantly reduced in their ability to cause tissue destruction in potato tubers (169). Unpublished data of Lingren *et* al. has demonstrated that the *slyA* homolog in *P. syringae* pv. *Tomato* is required for oxidative stress resistance and for the ability to cause disease on tomatoes. *RovA*, the *slyA* homolog of *Y. enterocolitica*, is required for virulence, regulation of invasion, and oxidative stress resistance (136, 153).

The importance of SlyA in the pathogenesis of *Salmonella* has been elucidated by several independent studies that have demonstrated that *slyA* mutants of *S.* Typhimurium are profoundly attenuated for virulence in the murine model of salmonellosis by the intravenous, intraperitoneal, and oral routes of infection (37, 119). Although these data suggest that *slyA* is required for invasion of the intestinal epithelium, analysis of *Salmonella* invasion in the murine model demonstrates that while *slyA* is required for survival in Peyer's patches and the destruction of M-cells, it is not required for the adherence to and invasion of M-cells (37). Although *slyA* is not required for invasion of M-cells, additional studies have demonstrated that *slyA* mutants of *S*. Typhimurium are unable to effectively replicate within the reticuloendothelial system of the host (119). The hallmark of *Salmonella* pathogenesis is the ability to survive within host phagocytes and, in particular, within host macrophages. The ability to cause lethal infection in mice requires survival within these cells and ultimately mechanisms of oxidative stress resistance, specifically, resistance to reactive oxygen species. Studies have found that *slyA* mutants are profoundly sensitive to *in vitro* derived oxidative stresses, namely hydrogen peroxide and paraquat, which suggests a role of SlyA in oxidative

stress resistance (119). In addition, a *slyA* mutant is unable to survive within elicited peritoneal macrophages in comparision to an isogenic wild-type strain (23).

Currently, little is known about the transcriptional regulatory properties of SlyA. Recent studies have shown that SlyA is required for the synthesis of proteins in *Salmonella* when grown to stationary phase or within macrophages (23). The observations that SlyA is essential for oxidative stress resistance, survival in macrophages, and virulence in mice suggests that there are several proteins that are either directly or indirectly regulated by SlyA that function to protect *Salmonella* from the toxic reactive oxygen species encountered within host phagocytes. The discovery of members of the SlyA regulon has been the focus of intense study in recent years and the identification of these genes will point to the role of SlyA in combating oxidative stress. Recent studies have demonstrated that SlyA can act as a repressor and activator of gene expression and altered levels of the proteins IroN, FliC, and PagC have been associated with a *slyA* mutation (164). These studies also demonstrated that SlyA binds to its own promoter suggesting the importance of tight SlyA regulation (164). Studies of Spory *et* al. (163) have determined that SlyA is responsible for the regulation of many proteins that are required for oxidative stress resistance. The evolutionary conservation of SlyA in a very diverse group of bacteria and archea suggest that SlyA plays a more global role in microbial survival in aerobic environments and that this protein is essential for *Salmonella* pathogenesis. Recent data of Norte *et* al (138) has determined that there is a region of the SlyA promoter that is positively regulated by PhoP and independent of the region that was found to be associated with SlyA binding (164). Furthermore, this study suggests that there may be an unknown factor through which PhoP exerts its effect (138). This finding only strengthens the idea that SlyA is an essential transcription factor

required for *Salmonella* virulence and that it may play a role with other known virulence factors. Future studies aimed at unraveling the members of the SlyA regulon will be instrumental in understanding how *Salmonella* are able to survive and replicate within the hostile environment of host phagocytes.

Conclusion

Salmonella infection continues to pose a worldwide health problem and the greatest health threat continues to be in the developing world. Although fatalities from *Salmonella* infection are not as significant as AIDS or malaria, economic losses due to sick leave approach the billions of dollars each year. *Salmonella* are unique pathogens that have the amazing ability to survive and replicate within host phagocytes. The evolution and acquisition of genes required for this lifestyle has been an essential aspect to their establishment of this environmental niche. *Salmonella* pathogenicity islands 1-5 all have been found to be required for some aspect of *Salmonella* pathogenesis, including survival within host phagocytes, invasion and penetration of the host epithelial cell layer lining the intestinal tract, or survival in the toxic environment of the stomach. Mutants that lack the expression of the genes encoded on all of these islands are attenuated for virulence in the mouse model of salmonellosis. The identification of several regulatory loci has also demonstrated their importance in *Salmonella* pathogenesis. The PhoP/PhoQ two-component system has been shown to be absolutely required for survival in host macrophages and for virulence in mice (54, 76, 131). The SlyA protein has also been shown to be absolutely required for oxidative stress resistance, survival in host macrophages and virulence in mice (23, 119). The interactions between an invading microorganism and its host are constantly changing and the continued adaptation of both the pathogen and its host is necessary for their survival.

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Chapter 2

The Ferritin-Like Dps Protein is Required for *Salmonella enterica* Serovar Typhimurium Oxidative Stress Resistance and Virulence

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Abstract

Resistance to phagocyte-derived reactive oxygen species is essential for *Salmonella enterica* Serovar Typhimurium pathogenesis. *Salmonella* can enhance its resistance to oxidants through the induction of specific genetic pathways controlled by SoxRS, OxyR, σ^S, σ^E, SlyA and RecA. These regulons can be found in a wide variety of pathogenic and environmental bacteria, suggesting that evolutionarily conserved mechanisms defend against oxidative stress both endogenously generated by aerobic respiration and exogenously produced by host phagocytic cells. Dps is a ferritin-like protein found in many eubacterial and archaebacterial species that appears to protect cells from oxidative stress by sequestering iron and limiting Fenton-catalyzed oxyradical formation. In *Escherichia coli* and some other bacterial species, Dps has been shown to accumulate during stationary phase in a σ^S-dependent fashion, bind non-specifically to DNA, and form a crystalline structure that compacts and protects chromatin from oxidative damage. In the present study, we provide evidence that Dps protects *Salmonella* during oxidative stress, promotes *Salmonella* survival in murine macrophages, and enhances *Salmonella* virulence in mice.

Introduction

Complex mechanisms have evolved to allow bacteria to withstand the oxidative stress associated with aerobic life (13). Many regulatory and enzymatic loci required for resistance to reactive oxygen species generated during aerobic metabolism have been found to play an important role in the ability of pathogenic bacteria such as *Salmonella enterica* serovar Typhimurium to cause disease (5, 8, 12, 15, 22, 24, 27, 28), suggesting that conserved strategies to resist oxidative stress in the environment can also allow organisms to withstand the respiratory burst of phagocytic cells.

Elegant studies of *Escherichia coli* by Kolter and colleagues first identified a low molecular weight protein that accumulates during stationary phase and binds to DNA; the protein was designated Dps, (DNA binding protein in stationary phase (2). Expression of *dps* in *E. coli* has been shown to be regulated by the stationary phase sigma factor RpoS (σ^{38}), OxyR, and IHF (3, 23). As *E.coli* enters stationary phase, more than 180,000 Dps molecules accumulate within a single organism, making Dps the most abundant protein in the cell (1). Dps-deficient mutant *E.coli* strains are unable to survive long term starvation (3, 17) and exhibit enhanced susceptibility to oxidative stress (24). *E. coli* Dps is able to form a microcrystalline structure on chromatin in intact stationary phase cells or in association with purified DNA *in vitro* (31), leading to the suggestion that Dps physically protects DNA by sequestration. However, the crystal structure of Dps has revealed similarity to the iron-storage protein ferritin (18, 32), and Zhao *et* al. (32) have found that Dps prevents DNA damage in *E. coli* through its capacity to bind Fe(II) and prevent the formation of hydroxyl radical. Recent studies in *Agrobacterium tumefaciens* and *Mycobacterium smegmatis*

indicate that Dps can prevent oxidative DNA damage even in the absence of DNA binding (10, 19).

The contribution of Dps to *Salmonella enterica* pathogenesis has not been investigated previously, although *dps* expression appears to be induced following *Salmonella* ingestion by macrophages (14, 29). In this study, we report the contribution of *dps* to *Salmonella* oxidative stress resistance, survival in macrophages, and virulence in mice.

Results and Discussion

The ability of *Salmonella* to survive and replicate within host phagocytes is absolutely essential for *Salmonella* virulence (16). Phagocyte-derived reactive oxygen species generated by the NADPH phagocyte oxidase play an important role in innate immunity to *Salmonella* (26, 30), and a number of *Salmonella* mutant strains with enhanced susceptibility to oxidative stress have a reduced capacity to survive in macrophages (9, 12, 23, 28). To determine whether *dps* is required for oxidative stress resistance, a *dps::aph* mutation was constructed in *S.* Typhimurium ATCC 14028s using the method of Datsenko and Wanner, where the *dps* open reading frame was deleted and replaced with the *aph* cassette (11). Oligonucleotide primers $^{5'}$TTAATTACCTGGGACACAAACATCAAG AGGATATGAGATTGTGTAGGCTGGAGCCTTC and $^{5'}$TACCTTCCTGCAACTCGAAG TATTCAGGGTAGAGATAGATATTCCGGGGATCCGTCGACC were utilized to create the *dps::aph* disruption, and primers $^{5'}$CGGTGCTATACTTATTTTCG and $^{5'}$CTGCGGATTCGCTGCGTTTG were used to confirm the expected insertion mutation. In addition, oligonucleotide primers $^{5'}$GCCAAAACTGAAGCTACAGGTGCCAAGTGC GCACTATGTCAGGAAACAGCTATGACCATG and $^{5'}$GAATGACCTCTTCCATCTTCC

ATCTCAGCGATCAGCGCGTCCGCTTTTACAACCAATAACCAATTC were used to create a *slyA::aph* disruption using the method of Datsenko and Wanner (11), and primers $^{5'}$GCTTTAGTTTTAGCCAAAACTG and $^{5'}$ACCGTCT CTCCACGCTAAAC were used to confirm the mutation. The absence of Dps protein in the *dps::aph* mutant strain was also confirmed by Western blot analysis using Dps-antisera (provided by R. Kolter, data not shown). Wild-type *S*. Typhimurium, the isogenic *dps::aph* mutant derivative (SL3474), the *dps::aph* mutant complemented with plasmid pBAD::Dps (SL3476), an isogenic *rpoS::*pRR10 (Δ*trfA*) mutant (SF1005) (15), and an isogenic *slyA::aph* mutant (SL3343) were compared for their susceptibility to various concentrations of hydrogen peroxide. Bacterial killing by hydrogen peroxide was measured in liquid medium as described by Buchmeier *et* al. (7). Briefly, overnight cultures were grown in LB medium, diluted to 10^6 CFU in phosphate-buffered saline (PBS) and incubated at 37^{o}C with a final concentration of 0.250 mM, 0.500 mM, or 1mM hydrogen peroxide. Aliquots were removed after 2 hours and the number of viable cells determined by serial dilution and plating onto LB agar. Percent survival following hydrogen peroxide challenge was calculated for each strain by dividing the number of colony forming units obtained from incubation in PBS alone by the number of colony forming units obtained from incubation in hydrogen peroxide. Each assay was repeated at least three times, and standard deviations were calculated and plotted. *Salmonella* carrying a *dps* mutation was found to be 15-fold less viable than wild type at 1 mM H_2O_2, and only slightly more resistant than a *slyA* mutant strain (Figure 1). The hydrogen peroxide sensitivity phenotype was complemented to near wild type levels by providing *dps* on a plasmid *in trans*. Thus, the role of *dps* in the ability of *S*. Typhimurium to withstand killing by hydrogen peroxide is comparable to observations in *E.coli* (25).

In order to determine the sensitivity of a *dps* mutant to intracellularly generated superoxide, we used the redox cycling agent, paraquat (20, 21), measured by the disc diffusion assay (4). Overnight cultures of wild type *S*. Typhimurium, SL3343 (*slyA*), SF1005 (*rpoS)*, SL3474 (*dps::aph*), and SL3476 (*dps::aph,* pBAD-Dps) were diluted in PBS and 10^6 cells were spread on M9 medium containing 0.5% glucose. Ten microliters of 10 μM, 100 μM, 1 mM, and 10 mM paraquat in sterile water was spotted on a paper disc placed in the center of the plate and plates incubated at 37°C overnight. The zone of growth inhibition was measured on two axes relative to the disc and averaged. A *dps* mutant is profoundly sensitive to paraquat as compared to wild type and can be complemented to near wild type resistance when *dps* is provided on a plasmid *in trans* as summarized in figure 2.

As discussed above, the antioxidant actions of Dps have been linked to its ability to sequester iron and prevent Fe (II)-dependent oxyradical formation. We therefore examined the ability of the Fe (II) chelator 2',2-dipyridyl to inhibit killing of *dps* mutant *Salmonella* by hydrogen peroxide. Twenty minute pre-incubation in 1 mM 2',2-dipyridyl completely rescued the *dps* mutant strain from killing by 2 mM H_2O_2, but provided only partial protection of *rpoS* or *slyA* mutant strains (Figure 3).

The intracellular survival of wild type and mutant *Salmonella* strains was determined in C3H/HeN (*ity*^{r}) primary peritoneal macrophages essentially as described previously (6, 30). Sodium periodate-elicited peritoneal macrophages were harvested from mice and plated at a density of 4-6x10^5/well. Macrophages were infected 24 hours later at a multiplicity of infection (MOI) of 5:1 (bacteria:macrophage) with wild type, *dps* mutant, or *slyA* mutant *S*. Typhimurium that were opsonized with normal mouse serum. Extracellular bacteria were killed by the addition of gentamicin (50 μg/ml) to the medium. Macrophages were lysed at

specific time points with 0.5% deoxycholate, serially diluted in PBS, and plated onto LB agar to determine the number of surviving bacteria. The percent-surviving bacteria were calculated from three separate experiments and averaged. The *dps* mutant strain was found to be impaired in its ability to survive in murine macrophages as compared to wild type (Figure 4), but not as severely as the *slyA* mutant strain. In addition, a *dps* mutant complemented by providing *dps in trans* shows partial restoration of survival inside murine peritoneal macrophages.

The virulence of *dps* mutant *S.* Typhimurium was assessed using a C3H/HeN (ity^R) murine model. Groups of four 8-week-old C3H/HeN ity^R female mice (Taconic Laboratories, Germantown, NY) were infected intraperitoneally with wild type, *dps* mutant or *slyA* mutant bacteria. Overnight cultures of bacteria were diluted phosphate-buffered saline and 200 µl of each dilution (approximately 1000 CFU) was administered intraperitoneally using a 25-gauge needle. The inoculum size was confirmed by serial dilution and plating onto LB agar (data not shown). Infected mice were monitored for survival during a two-week period. Only one of four mice infected with the *dps* mutant died during the course of the experiment and the remaining mice never showed signs of illness. The virulence assay was repeated several times, with virtually identical results. All mice infected with wild type *Salmonella* succumbed by 9 days post infection, but no mice infected with the *slyA* mutant strain died. These results demonstrate the crucial importance of *dps* in the ability of *S.* Typhimurium to cause lethal infection in mice.

As intraperitoneal lethality is not the optimum test for virulence since it is measuring the cumulative growth, survival, and then the lethal aspects of the bacteria, quantification of bacteria from mouse tissues was performed. Groups of four mice were infected with 1800

CFU of wild type *S.* Typhimurium 14028s, a *dps* mutant, and a *dps* mutant complemented by providing *dps in trans*. After five days the mice were euthanized, the spleens and livers were recovered, and homogenized in three milliliters of sterile water. Enumeration of viable bacteria from each organ was determined by serial dilution in PBS and plating onto XLD agar. The results are illustrated in Figure 6. These data demonstrate that Dps is required for survival and the attenuation of virulence that is seen in C3H/HeN mice is due to a defect in intramacrophage survival. A *dps* mutant complemented by providing *dps in trans* shows a partial recovery in the number of surviving bacteria in the spleen and liver.

Resistance to oxidative stress plays an important role in the ability of *Salmonella* to resist killing by host phagocytes and cause a productive infection. Pathogenic microorganisms can resist host-derived reactive oxygen species by avoidance, inhibiting production, production of scavengers, metabolic detoxification, or repair of damage. Iron sequestration is another important mechanism, since intracellular Fe (II) can catalyze the formation of highly toxic oxyradicals from hydrogen peroxide.

The ferritin-like protein Dps has been found in many eubacterial and archaebacterial species. Similar to ferritin, Dps can sequester iron atoms to prevent their participation in the formation of toxic reactive oxygen species (32). Additionally, some Dps homologues appear to condense chromatin into a microcrystalline array that may physically protect DNA from damage (31). In the pathogenic gram-negative bacterium *Salmonella*, *dps* expression is induced following internalization of the bacteria by macrophages (14, 29). The results of our studies unequivocally demonstrate a role of *S.* Typhimurium *dps* in oxidative stress resistance and virulence. The most likely mechanism is the sequestration of iron and prevention of iron-dependent oxidative DNA damage. Dps can be added to the list of

evolutionarily conserved antioxidant proteins employed by *Salmonella* to resist killing by host phagocytes.

Acknowledgements

We thank G. Thomas, A. Treece, N. Borden, H. Hassan, and B. J. Welker for their technical assistance, and R. Kolter for insightful suggestions strains, plasmids, and polyclonal Dps antibody. This work was supported by NIH grants to S.J.L (AI48622) and F.C.F. (AI50660).

Figure 1. *dps* mutant S. Typhimurium has enhanced susceptibility to hydrogen peroxide. Results are expressed as percent survival after 2-hour exposure to 0.250 mM, 0.500 mM, or 1.0 mM hydrogen peroxide at 37^{o}C and represent the average percent survival from three independent assays. Error bars represent the standard deviation of three independent experiments. *$p<0.01$ by Student's t test as compared to wild type.

Figure 1.

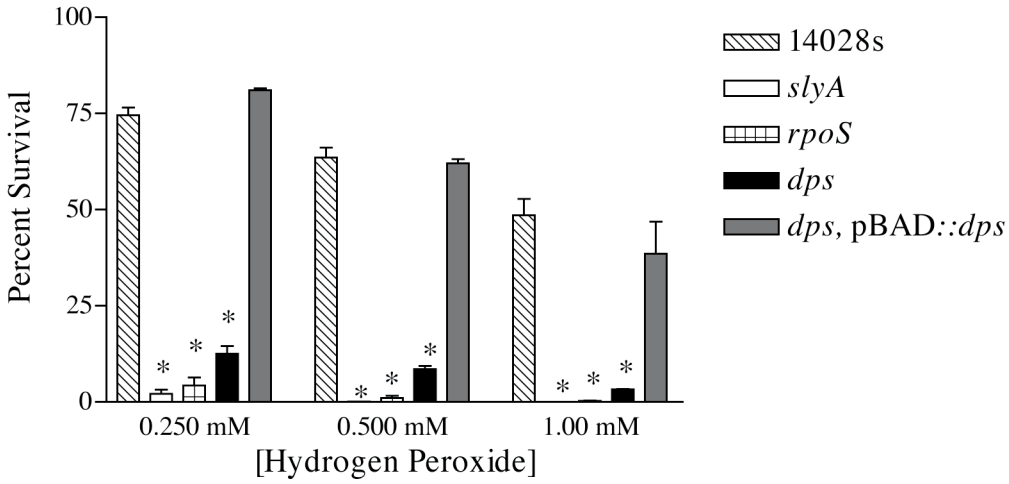

100
75
50
25
0
Percent Survival
*
0.250 mM
0.500 mM
1.00 mM
[Hydrogen Peroxide]
14028s
slyA
rpoS
dps
dps, pBAD::dps

Figure 2. *dps* mutant *S.* Typhimurium is sensitive to intracellular generated superoxide. Strains were tested for their susceptibility to 0.01 mM, 0.1 mM, 1.0 mM, or 10.0 mM paraquat. The zone of inhibition was measured in millimeters in four axes and the results are expressed as the average of three independent assays with error bars representing the standard deviation for each. Wild type (SL3202) sensitivity to paraquat is shown as hatched bars, data from the *slyA* mutant (SL3343) is represented as open (white) bars, sensitivity of the *rpoS* mutant (SF1005) is shown as double-hatched bars, data for the *dps* mutant (SL3474) is shown as filled bars (black), and paraquat sensitivity of the *dps* complement (SL3476) is represented as grey bars.

Figure 2.

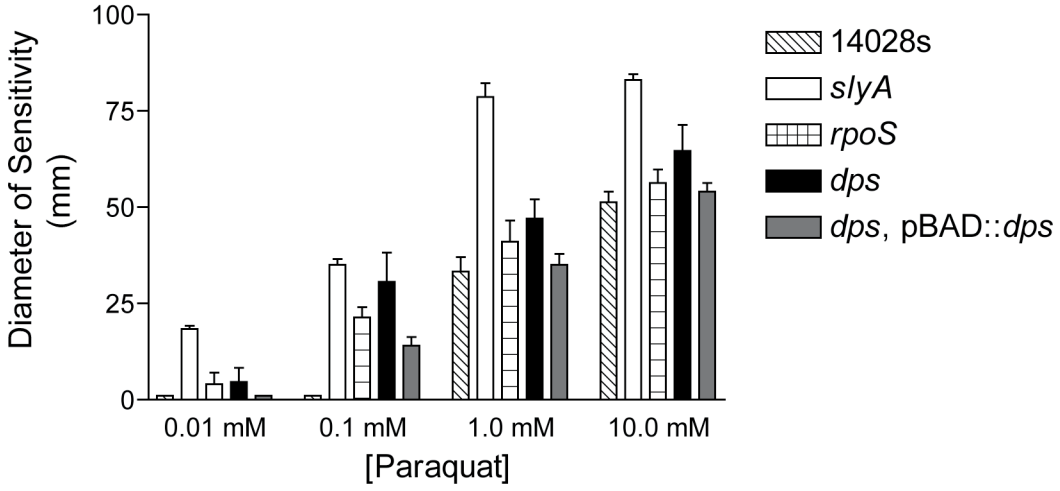

Diameter of Sensitivity (mm)
100
75
50
25
0
0.01 mM
0.1 mM
1.0 mM
10.0 mM
[Paraquat]
14028s
slyA
rpoS
dps
dps, pBAD::dps

Figure 3. The iron chelator, 2',2-dipyridyl, rescues *dps* mutant *Salmonella* challenged with hydrogen peroxide. Each strain was pretreated with 1 mM 2',2-dipyridyl or PBS for 20 minutes and then incubated with 2 mM H_2O_2 for 20 minutes at 37^{o}C. Viable bacteria were determined by serial dilution and plating on LB agar. The numbers of surviving bacteria are expressed as a percentage of the original inoculum. The average percent survival from three independent experiments is shown, with error bars indicating standard deviation. *$p<0.05$ and **$p<0.001$ by Student's t test as compared to wild type.

Figure 3.

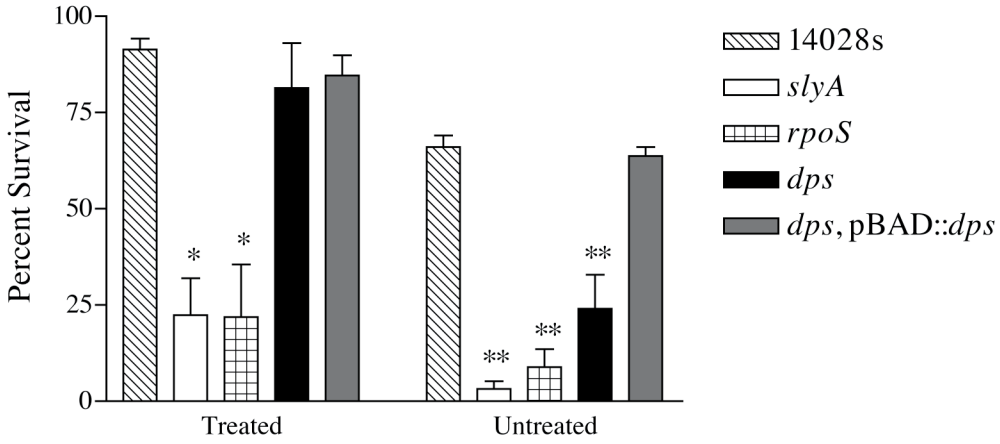

Percent Survival
100
75
50
25
0
*
*
**
**
**
Treated
Untreated
14028s
slyA
rpoS
dps
dps, pBAD::dps

Figure 4. *dps* mutant *Salmonella* exhibits reduced survival in periodate-elicited murine peritoneal macrophages. Survival of *Salmonella* strains was determined in C3H/HeN (ity^R) peritoneal macrophages at 3, 6 and 12 hours post-infection. Murine peritoneal macrophages were elicited with 5 mM sodium periodate and harvested 4 days later. Macrophages were seeded at approximately $5x10^5$ macrophages per well in 48-well plates and infected with *Salmonella* strains at a multiplicity of infection (MOI) of 5:1 (bacteria:macrophages). Results are expressed as percent survival and represent the average of two independent assays, with error bars indicating standard deviation. *$p<0.05$ by Student's t test as compared to wild type.

Figure 4.

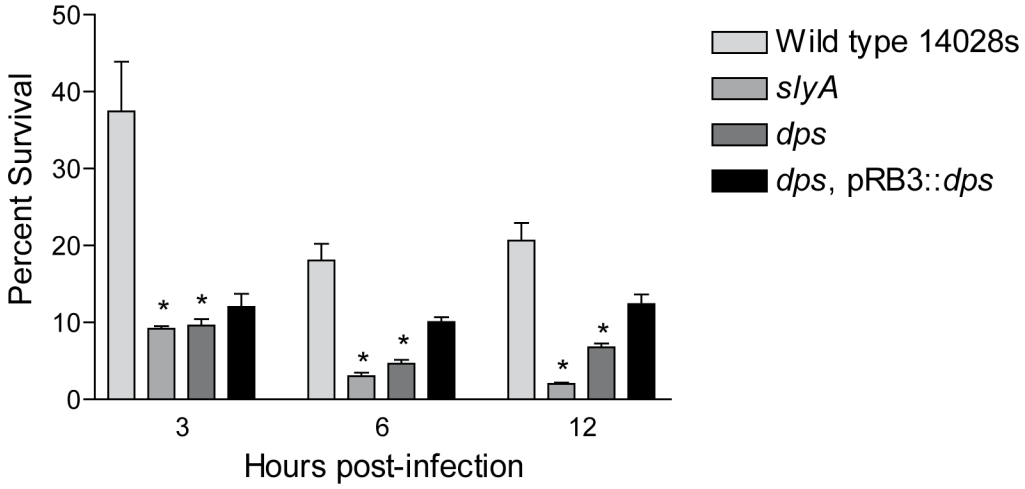

Percent Survival
50
40
30
20
10
0
3
6
12
Hours post-infection
Wild type 14028s
slyA
dps
dps, pRB3::dps

Figure 5. *dps* mutant *Salmonella* is attenuated for virulence in C3H/HeN (ity^R) Mice. Groups of four 8-week-old C3H/HeN female mice (Taconic Laboratories) were infected intraperitoneally with wild type (14028), an avirulent *slyA* mutant (SL3343), and a *dps* mutant strain (SL3474). Approximate 1000 CFU of each strain in phosphate-buffered saline was administered intraperitoneally. The survival of infected mice from three independent experiments is shown. *$p<0.05$ by Student's t test as compared to wild type.

Figure 5.

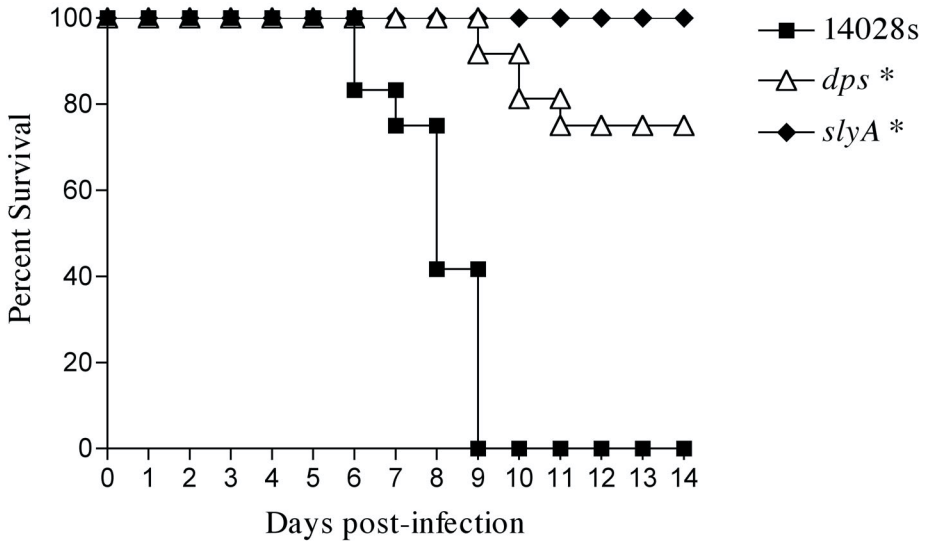
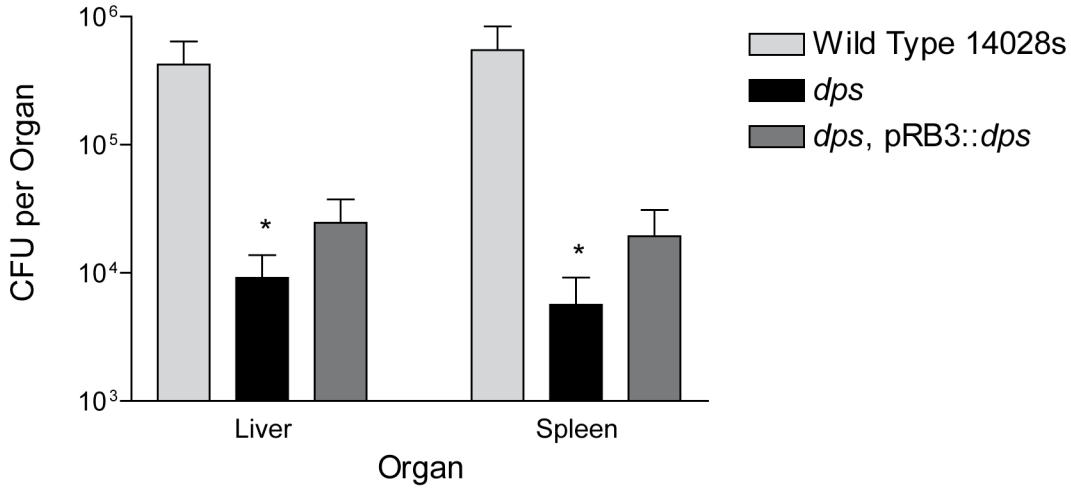

Percent Survival
100
80
60
40
20
0
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14
Days post-infection
14028s
dps *
slyA *

Figure 6. *dps* mutant *Salmonella* showed reduced survival *in vivo*. Groups of four mice were infected intraperitoneally with 1500 CFU of wild type *S.* Typhimurium, a *dps* mutant, and a *dps* mutant complemented by providing *dps in trans*. After 5 days, the mice were euthanized and the spleens and livers were recovered. The organs were homogenized in three milileters of sterile water, serially diluted in PBS, and plated onto XLD agar to enumerate viable bacteria per organ. The results are expressed as CFU/organ and error bars are included to represent standard deviations. *$p<0.02$ as determined by Student's T-Test as compared to wild type.

Figure 6.


10^6
10^5
10^4
10^3
CFU per Organ
*
*
Liver
Spleen
Organ
Wild Type 14028s
dps
dps, pRB3::*dps*

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Chapter 3

The Role of *slyA* in Oxidative Stress Resistance in *Salmonella enterica* Serovar Typhimurium

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Abstract

Periods of rapid growth and prolonged starvation are common among bacteria within their natural environments. Bacterial adaptation to the stresses encountered during these times must occur in order to ensure their survival. In *Salmonella enterica* Serovar Typhimurium, at least 60 genes have been identified whose expression is increased in response to oxidative stresses. One of these genes, called *slyA*, and the genes regulated by it, are needed for survival in host macrophages and for surviving oxidative stress conditions. In this study, the role of the SlyA regulon in *Salmonella* survival during long-term growth conditions was examined. Various double mutations were constructed that inactivated regulons know to be required for oxidative stress resistance, such as *oxyR, dps, arcA, FNR, cpxR, rpoE, rpoS,* and *soxRS*. We have found that certain mutations are extremely detrimental to the ability of *S.* Typhimurium to survive aerobic growth conditions, while the ability to survive anaerobic growth conditions was unaffected. Mutants lacking *slyA* and either *rpoE, rpoS,* and *soxRS* showed significantly reduced survival rates under prolonged aerobic growth conditions. These double mutants demonstrated greater susceptibility to hydrogen peroxide and paraquat than did wild type or the single mutants alone. Reduced growth rate and survival of the *slyA*, *slyA/rpoE, slyA/rpoS,* and *slyA/soxRS,* was restored to near wild type levels when cells were grown anaerobically. These data suggest that the mechanism of protection afforded by the SlyA regulon against oxidative stresses may be separate from other oxidative stress regulators.

Introduction

Although oxygen is essential for the survival of all aerobic organisms, mechanisms of defense against the toxic by-products of aerobic metabolism are equally important for survival. Bacteria are exposed to oxidative stress during periods of prolonged starvation, as a consequence of aerobic metabolism and also within professional phagocytes. Reports have demonstrated that reactive oxygen species do accumulate during stationary phase and studies of Gort *et* al. (22) show periplasmic superoxide is produced during stationary phase and, without an oxidative resistance response, damage done by hydrogen peroxide is accelerated. As a result, many bacteria have evolved defense mechanisms against these species to ensure their survival in these hostile environments. Recent studies have shown that the sigma factors, σ^{E} and σ^{Σ}, are both required for *Salmonella* resistance to oxidative stress and for prolonged stationary phase survival (18, 45). Survival can be restored in these mutants when grown anaerobically. These results indicate that the antioxidant functions of proteins under stationary phase control are needed for stationary phase survival and, not surprisingly, these same genes are also needed to survive in host phagocytes.

The ability of *Salmonella* to survive and replicate in host immune cells is a hallmark of this pathogen. *Salmonella* existence within the phagosomal compartment of the macrophage requires resistance to an onslaught of reactive nitrogen and oxygen species as the result of the phagocyte respiratory burst. The genes and the control mechanisms required for surviving phagocyte derived oxidative stress have been extensively studied in *Salmonella* and requires the coordinate regulation of a large number of genes (1, 2, 7, 25). Varied patterns of *Salmonella* gene expression have been demonstrated during logarithmic and stationary phases of growth, and it has been proposed that stationary phase may more closely

parallel conditions encountered within host cells (11). Surprisingly, the only genes that have a demonstrated role in resistance to *in vitro* derived oxidative stress conditions and virulence in mice are: *rpoS*, *zwf* (glucose-6-phosphate dehydrogenase), *slyA*, *sodC*, *rpoE*, *recA*, and *recBC* (6, 8, 9, 15, 18, 27, 28, 30, 45).

A regulatory protein, called SlyA, has been identified in a large and diverse number of bacteria. The *slyA* gene was first recognized as a gene that confers a hemolytic phenotype on *Escherichia coli* K-12 when the gene is supplied on a plasmid *in trans*. Further work has demonstrated that *slyA* is required for the virulence of *S.* Typhimurium in mice and for the survival in murine peritoneal macrophages (6, 28). Although *slyA* was first presumed to encode a hemolytic protein, recent studies have demonstrated that *slyA* is a transcriptional regulator that induces the expression of a 34 kDa pore-forming protein, *clyA*, in *E. coli* (29, 34). A large number of genes have been found to be regulated either positively or negatively by SlyA; however, many have yet to be identified. Recently, the studies of Spory *et* al. (42) identified a number of SlyA-controlled proteins by mass spectrometry that proved to be stress response proteins. These studies confirm previous findings that SlyA regulates the synthesis of several proteins during stationary phase and during infection in macrophages that may be involved in oxidative stress resistance (6). Although recent reports have demonstrated that survival of a *slyA* mutant grown in minimal medium is reduced (43), the importance of SlyA for survival during prolonged stationary phase conditions and its role in oxidative stress resistance has yet to be elucidated.

The long term stationary phase survival assays presented here suggest that SlyA is essential for survival in oxygen rich environments. When the same mutants are assayed in the absence of oxygen, survival can be restored to near wild type levels. This work also

demonstrates that a *slyA* mutant is hypersusceptible to reactive oxygen species and that by supplying *slyA in trans*, resistance to oxidative stress can be restored to near wild type levels. These studies also show that the SlyA regulon functions independently of other known oxidative stress regulators and that *slyA* expression can be induced under environmental conditions that closely resemble those conditions found within professional phagocytes. These results indicate that SlyA functions as a regulator of a number of genes required for prolonged stationary phase survival and oxidative stress resistance. The complex and intricate balance of gene regulation at the transcriptional level is essential for bacterial survival during conditions of nutrient starvation and oxidative stress, involving complex programs of gene expression. It has previously been shown that *slyA* is maximally expressed in stationary phase independent of *rpoS* and also within macrophages. The studies presented here also reveal the identification and characterization of a novel regulatory locus that is required for the expression of *slyA in vitro*. This 7-kilobase region of DNA comprises 6 open reading frames and is unique to *Salmonella* species. The transcriptional regulation of SlyA by this locus may induce a SlyA-dependent pathway that enables *S.* Typhimurium to survive and replicate within host phagocytes and consequently cause disease.

Results

slyA mutant Salmonella are hypersusceptible to reactive oxygen species

Although previous studies have demonstrated that SlyA is essential for oxidative stress resistance (6), the frequency of reversion of this mutant (SL2236) to a wild type genotype prevented the prolonged stationary phase survival studies of this mutant. A new *slyA* mutant was created by gene replacement using the method of Datsenko and Wanner (14)

in order to study the role of SlyA during prolonged stationary phase. The susceptibility of *slyA Salmonella* to reactive oxygen species was investigated by incubating bacteria in the presence of various concentrations of H_2O_2 and the redox cycling compound, paraquat. After incubation in hydrogen peroxide, bacteria were serially diluted in phosphate-buffered saline and plated onto LB agar to identify viable bacteria. Following a two hour incubation with as little as 250 μM hydrogen peroxide resulted in a >100-fold reduction in the number of *slyA Salmonella*, whereas little killing of the wild type or a *slyA* mutant containing a functional *slyA* gene provided *in trans* was observed (Figure 1a). Additionally, *slyA Salmonella* are profoundly sensitive to paraquat, which generates intra- and extracellular superoxide anion, whereas, a complemented strain demonstrates sensitivity comparable to wild type (Figure 1b). At a paraquat concentration as low as 10 μM, a *slyA* mutant shows an 18.6 mm diameter of sensitivity while the wild type and *slyA* mutant complemented by providing *slyA in trans* show no sensitivity at this concentration. These results indicate that *slyA* is required for *in vitro* resistance to oxidative stress.

slyA mutant S. Typhimurium has reduced viability during prolonged stationary phase under aerobic conditions, but not anaerobic conditions

In order to investigate whether *slyA* is important for survival under aerobic growth conditions, growth rate studies were performed under both aerobic and anaerobic growth conditions. Bacteria were grown at 37^{o}C with aeration overnight in LB broth. For anaerobic growth rate studies, bacteria were diluted 1:1000 in fresh LB supplemented with 0.5% glucose and grown in an anaerobic chamber. Aerobic growth rate studies were performed by diluting bacteria to an OD_{600} = 0.1 and incubating the cultures in a Bioscreen C (Labsystems)

incubator. These studies revealed that *slyA* mutant *Salmonell*a exhibit a prolonged lag phase during growth under aerobic conditions (Figure 2a), while a *slyA* mutant grows at a rate comparable to wild type under anaerobic growth conditions (Figure 2b). A *slyA* mutant grows at a rate of 0.06 $hours^{-1}$ during the first five hours in culture, while the wild type and *slyA* mutant complemented by providing *slyA in trans* grow at rates of 0.84 $hours^{-1}$ and 0.41 $hours^{-1}$ respectively. After ten hours in culture, however, the *slyA* mutant grows at a rate that is comparable to wild type and the *slyA* complement (0.39 $hour^{-1}$, 0.84 $hours^{-1}$, and 0.41 $hours^{-1}$ respectively). Under anaerobic growth conditions, the *slyA* mutant grows at rate that is comparable to both wild type and the *slyA* complement (1.38 $hours^{-1}$, 1.66 $hours^{-1}$, and 1.68 $hours^{-1}$ respectively). This data suggests that *slyA* may serve a protective function from oxidative injury encountered as a result of aerobic metabolism.

Prolonged stationary phase survival includes bacterial exposure to toxic metabolites generated as a consequence of aerobic metabolism (20). Elegant studies of Testerman *et* al. (45) have demonstrated that the accumulation of reactive oxygen species during prolonged stationary phase cause a *rpoE/ropS* double mutant to self-sterilize within 24 hours under aerobic culture conditions. Under prolonged stationary phase conditions, the *slyA* mutant shows profound loss of viability (< 1%) after just 24 hours in aerobic stationary-phase conditions and a complete loss of viability after just 5 days in continuous aerobic culture (Figure 3a), while this phenotype is absent during continuous culture under anaerobic conditions (Figure 4a). Mutations in other known oxidative stress loci do not confer this phenotype, thus demonstrating that the SlyA regulon is regulated independently from these oxidative stress genes. In addition, the oxidative stress phenotype of a *slyA* mutant can be complemented by *slyA* provided *in trans* to survive as well as the wild type strain under

aerobic conditions (Figure 3a). Furthermore, combining a *slyA* mutation with *rpoE*, *rpoS*, or *soxRS* mutations results in profound loss of viability during prolonged stationary phase under aerobic conditions (Figure 3b), however survival is restored in these mutants under anaerobic conditions (Figure 4b).

Regulation of slyA::lacZ Expression

Previous reports have shown that *slyA* is maximally expressed in stationary phase independent of *rpoS* and also within the phagosomal compartment of macrophages (6). The contribution of other known oxidative stress regulators including *phoP*, *rpoS*, *rpoE*, *oxyR*, *soxRS*, or *relA* to *slyA* expression has not yet been examined. In order to examine their contribution we combined defined mutations by P22-mediated transduction in each of these regulators with a *slyA*::*lacZ* merodiploid to examine how *slyA* expression is influenced by any of these regulators. The results of these studies demonstrate that *slyA*::*lacZ* expression is unaffected when combined with defined mutations in other known oxidative stress regulators such as *phoP*, *rpoS*, *rpoE*, *oxyR*, *soxRS*, or *relA* (Figure 5). These data suggest that SlyA functions independently of these other known regulators.

Although there are no clear studies demonstrating the conditions found within the phagosomal compartment of macrophages, reports by Eriksson *et* al. (17) suggest that the environment is acidic in pH and low in nutrients. In order to determine if *slyA* expression is influenced by these conditions, we measured *slyA*::*lacZ* expression when grown under various media conditions. Our data show that *slyA*::*lacZ* expression can be induced when grown in minimal medium containing low Mg^{2+} (8 μM) (Table 2). We also found that *slyA*::*lacZ* expression is maximal (5-fold induced) when grown under conditions of low Mg^{2+} and low pH (5.7). These conditions are also the conditions that have been reported to induce

the PhoP/PhoQ two-component regulatory system (10, 21, 23, 24, 32, 40). The contribution of PhoP to *slyA* expression will be discussed in more detail below.

SlyA expression is autoregulated only when in a phoP-constiutive mutant background and slyA is provided in trans

Reports by Green *et* al. (43) suggest that SlyA can negatively regulate its own expression. These studies were performed using a transcriptional *slyA*::*lacZ* fusion that was encoded on a high copy plasmid. To further investigate this observation, we used a chromosomal *slyA::lacZ* merodiploid reporter fusion and provided *slyA in trans* to determine if *slyA* could negatively regulate its own expression. These studies were performed by supplying *slyA in trans*, where transcription of *slyA* was under the control of an arabinose inducible promoter (pBAD::*slyA*). The results of these experiments show that *slyA::lacZ* expression is not significantly affected by increased *slyA* provided *in trans* (Table 3). Our data demonstrate that *slyA*::*lacZ* expression can be reduced by supplying *slyA in trans* only in the background of a *phoP*-constitutive mutant as illustrated in Figure 6. The *phoP*-constitutive mutant has a point mutation in amino acid 24 of the *phoQ* protein that results in constitutive phosphorylation (activation) of PhoP and subsequent activation of the PhoP regulon. In a *phoP* background, *slyA::lacZ* expression is not significantly affected. This finding was corroborated by real time (quantitative) PCR, where *slyA* transcript was quantified in wild type, a *phoP,* and a *phoP*-constitutive background (Figure 7). Green *et* al. (43) proposed that PhoP can interact with a portion of the *slyA* promoter. The region of this interaction of PhoP with the SlyA promoter is upstream of the previously described SlyA associated portion of this same promoter (33, 43). Although these studies suggest that PhoP

directly affects *slyA* expression by physical association with DNA at the *slyA* promoter, EMSA and DNase I protection assays of the promoter region with purified PhoP protein have yet to be demonstrated.

Identification of a novel regulatory locus that is required for slyA::lacZ expression

Our previous data have demonstrated that *slyA* expression is not significantly affected by other known oxidative stress regulators. A classical transposon mutagenesis study was performed to identify loci that affect *slyA* expression. This was done using the method of Rappleye and Roth (36). Briefly, a Tn*10*dTet element was transduced into a strain encoding a chromosomal *slyA::lacZ* fusion and the transposase was provided *in trans* on plasmid pNK972. Transductants were plated onto LB agar containing 20 μg/mL tetracycline and 50 μg/mL X-gal. Colonies that were dark blue (hyper-induced *slyA* expression) or white (hyper-repressed *slyA* expression) were used for subsequent analysis. As a result of this study we have identified a 7-kilobase region of DNA comprising 5 open reading frames encoding two putative amino acid transporters, a putative two-component regulatory protein, a putative decarboxylase, and a putative cytoplasmic protein that is present only in *Salmonella* and is totally absent from *E. coli* (Figure 8). This region of DNA is inserted between *ubiX* and *purF* that are located at centisome 51.3 and centisome 51.7 of the *Salmonella* chromosome respectively. The transposon insertion within STM2359 completely abolishes *slyA::lacZ* expression (Figure 5). The phenotypic characteristics of this mutant are similar to a *slyA* mutant suggesting that this region of DNA is important for SlyA expression. The sensitivity of this mutant to hydrogen peroxide is comparable to a *slyA* mutant (2-fold greater) and shows reduced survival as compared to wild type (> 3.5-fold). These data are illustrated in

Figure 9. The virulence of this mutant was also tested in C3H/HeN mice. The results of this experiment show that only 1 mouse succumbed to lethal infection suggesting that this transposon insertion does indeed abolish *slyA* expression (Figure 10). Although we would expect all of the mice to survive that are infected with this transposon mutant if *slyA* expression has been completely abolished, our data suggest that *slyA* expression could be induced by an independent mechanism. Collectively, these data suggest that this region of DNA is essential for *slyA* expression and could also explain why *slyA* mutants of *E. coli* K-12 strains, which are missing these five open reading frames, do not demonstrate the same phenotypic characteristics of *slyA* mutant *Salmonella*.

Discussion

Survival during prolonged stationary phase presents a number of challenges for bacteria that includes nutrient limitation, the accumulation of toxic metabolites, oxidative injury, and alterations of pH (20, 41). In this study we present evidence that *slyA* is required for survival under these conditions. Expression of *slyA* seems to be even more important for survival under these conditions than the expression of other known oxidative stress regulators or stationary phase required genes. The aerobic survival and growth rate defect of a *slyA* mutant *S*. Typhimurium can be rescued when grown anaerobically. Under anaerobic conditions a *slyA* mutant has a growth rate comparable to that of wild type suggesting that oxidative injury is the primary reason for decreased viability during prolonged stationary phase.

Extensive studies have been done to investigate the role of the alternative sigma factors, σ^S and σ^E, in starvation survival. A mutation in either sigma factor cause cells to

show decreased viability under aerobic starvation conditions while a mutant lacking both σ^S and σ^E is completely non-viable after just 24 hours in long-term culture (45). Our results demonstrate that the combination of a *slyA* mutation with *rpoS* or *rpoE* results in a significant loss of viability under aerobic starvation conditions while complete abrogation of this phenotype can be observed under anaerobic conditions (Figure 3). Furthermore, a single mutation in *rpoS* or *rpoE* caused a slightly reduced viability that is comparable to wild type (Figure 3). These data suggest that the SlyA regulon may function independently of these regulons during prolonged starvation and that the SlyA regulon may be comprised of other oxidative stress resistance genes.

Regulation of gene expression at the transcriptional level is an important mechanism by which bacteria can control protein synthesis. It has been suggested that SlyA may be under the control of other known oxidative stress regulators. From the studies presented here, SlyA expression is only affected by a transposon insertion into STM2359, when grown in conditions that are postulated to be those found within the phagosomal compartment of macrophages, or in the background of a *phoP*-constitutive mutant. Collectively, these data suggest that SlyA is global transcriptional regulatory protein that may be under the control of several independent pathways that are induced by different environmental signals. The PhoP/PhoQ two-component system is essential for intramacrophage survival and for systemic infection in mice and the observation that SlyA may be activated by this system only strengthens the idea of a coordinated response in gene expression involving several transcription factors mounting an appropriate response to environmental stimuli.

The isolation of a transposon insertion into STM2359 has allowed for the identification of a novel region of DNA that is essential for *slyA* expression. Phylogenetic

analysis of this region suggests that Salmonella may have acquired this region of DNA after its divergence from *E. coli* and that this region is important in *Salmonella* pathogenesis. The exact role of the *slyA* gene in *E. coli* K-12 strains has not been elucidated, however, it may play a different role in this bacterium. Although, our data demonstrate that a transposon insertion into STM2359 completely abolishes *slyA* expression, further studies are needed to illustrate the exact mechanism of how these proteins activate *slyA* expression. One possibility is that the putative two-component regulatory protein found within this region directly binds to and activates that *slyA* promoter, however, there could also be an indirect effect where this protein activates an intermediate factor between the two proteins. Once these studies have been performed more will be known about the interplay between several independent transcription factors that can direct the activation of the global transcription factor, SlyA.

In conclusion, the work presented here demonstrates that SlyA is a global transcription factor that is essential for survival within an aerobic environment. These observations suggest a direct link between the PhoP/PhoQ two-component system and the transcriptional regulator, SlyA; however, the exact mechanism of this interaction is still unknown. We also demonstrate that the novel regulatory locus that is only found in *Salmonella* spp. and is absent in *E. coli*. This locus is required for *slyA* expression and a transposon insertion mutant in STM2359 shows increased sensitivity to hydrogen peroxide and attenuation for virulence in mice comparable to a *slyA* mutant. The complex regulatory network of gene expression presented here only demonstrates that the interplay and overlap between pathways enables *Salmonella* to mount an appropriate cellular response depending on the environmental conditions.

Experimental Procedures

Media and growth conditions

Bacteria were grown in Luria-Bertani (LB) medium with or without 0.5% glucose at 37ºC either aerobically or anaerobically. Aerobic cultures were agitated at 200 r.p.m while anaerobic cultures were grown statically. LB was supplemented with 200 mg ml^{-1} penicillin, 20 mg ml^{-1} tetracycline, 50 mg ml^{-1} kanamycin, or 25 mg ml^{-1} chloramphenicol from Sigma as appropriate. Evans blue uranine medium was used to identify pseudolysogen-free transductants when constructing double mutants (5). LB medium supplemented with 10 mM EDTA was used to prevent phage re-infection. For *slyA::lacZ* induction studies, cultures were grown in one of the following medias: LB adjusted to pH 5, 6, or 7, LB with the addition of 10 μM, 100 μM, or 1 mM H_2O_2, N minimal media (38, 39) adjusted to pH 4.5 or 7.5 and supplemented with 0.1% casamino acids and 38 mM glycerol, with 8 μM or 200 μM $MgCl_2$, MOPS supplemented with 0.2% and 5 % glucose, or M9 minimal media supplemented with 0.2% glucose and 10 μM or 1 mM $MgCl_2$.

Bacterial strains and plasmids

All strains are derivatives of *Salmonella enterica* serovar Typhimurium ATCC 14028s or isogenic derivatives (Table 1). For the construction of *S*. Typhimurium SL3343 (*slyA::aph*), the kanamycin cassette was amplified from pUC-4K (Pharmacia) plasmid DNA using primers 5'-GCCAAAACTGAAGCTACAGGTGCCAAGTGCGCACTATGTCAGGAAACAGCTATGACCATG and 5'-GAATGACCTCTTCCATCTCAGCGATCAGCGCGTCCGCTTTTACAACCAATTAACCAATTC, which are both flanked with 40-nucleotide extensions homologous to regions upstream and downstream

of the *slyA* open reading frame. The *slyA* gene was replaced by the kanamycin cassette using the phage lambda gamma-beta-exonuclease (Red recombinase) expression plasmid pKD46 as described by Datsenko and Wanner (14). Briefly, wild-type *S*. Typhimurium containing the pKD46 plasmid was made competent by growing the cells to OD_{600} of 0.6 in the presence of 1mM L-arabinose to induce the expression plasmid. The cells were then washed 3-4 times in ice-cold 10% glycerol, transformed with 5 μl of purified DNA (0.5 mg ml^{-1}), suspended in 1 ml SOC, and incubated at 37°C for 1 hour with aeration. Bacteriophage P22 lysate was made on 5 colonies and transduced into wild-type *S*. Typhimurium 14028s. EBU medium was used to identify pseudolysogen-free transductants. Replacement of the *slyA* gene with the kanamycin cassette was confirmed by PCR and Southern hybridization creating strain SL3343. For the construction of the *slyA* complementing strain, *slyA* was amplified from the chromosomal DNA of wild type *S*. Typhimurium 14028s and cloned into the *EcoRV* site of the plasmid pRB3. This plasmid was then transformed into SL3343 by electroporation creating strain SL3383. For the construction of double mutants, P22 bacteriophage lysate was made on each of the strains harboring the mutations of other known oxidative stress regulators. An overnight broth culture of the *slyA* mutant was then infected with the bacteriophage lysate at multiplicity of infection of 5:1, plated on LB agar with the appropriate antibiotics, and grown overnight at 37°C under both aerobic and anaerobic conditions. Evans blue uranine medium was used to identify pseudolysogen-free transductants. For the expression studies, mutations were made using P22-mediated transduction into the chromosomal *slyA::lacZ* merodiploid (SL2741).

Long-term stationary phase survival assays

Culture tubes containing 10 ml LB supplemented with antibiotics as indicated were inoculated with 10 mL of bacteria from overnight cultures. For anaerobic cultures, LB medium was supplemented with 0.5% glucose. For anaerobic survival assays, tubes were incubated at 37^{o}C in a Coy Anaerobic Chamber B (Coy Laboratory Products Inc.) without aeration. Aerobic cultures were grown at 37^{o}C with aeration at 200 r.p.m. Aliquots were removed daily, serially diluted in phosphate-buffered saline and plated on LB agar for quantification of colony-forming units.

β-Galactosidase and oxidative stress sensitivity assays

Quantification of β-galactosidase activity used a modification of the assay previously described (31, 37). Briefly, 100 μl of culture aliquots were used for each experiment and assays were incubated at 28^{o}C to ensure uniformity. Each assay was performed in triplicate and the experiments were repeated three times. Hydrogen peroxide assays were performed by incubating 10^{6} CFU ml^{-1} of an overnight culture with 0.125 mM, 0.250 mM, 0.375 mM, 0.500 mM, and 1 mM concentrations of hydrogen peroxide in phosphate-buffered saline at 37^{o}C for two hours. The cells were then serially diluted in PBS and plated on LB agar for quantification of colony-forming units. The sensitivity of a *slyA* mutant to the intracellularly generated superoxide anion, we used the redox cycling agent, paraquat (methyl viologen; Sigma), in a disc diffusion assay (3). The assay was performed using the protocol of Buchmeier *et* al. (4). Overnight cultures of strains *S*. Typhimurium 14028s, SL3343, and SL3383 were diluted in phosphate-buffered saline, and 10^{6} cells were spread on M9 medium containing 0.5% glucose and allowed to dry. Ten microliters of 10 μM, 100 μM, 1mM, and

10 mM paraquat made in sterile water was spotted on a paper disc placed in the center of the plate and incubated at 37^{o}C overnight. The zone of growth inhibition was measured on two axes relative to the disc and averaged.

Transposon (T-POP) Mutagenesis

A strain containing a chromosomal *slyA*::lacZ fusion (SL2741) was electroporated with pNK972 (P_{tac}-*tnpA*$^{+}$) and transformants were selected for on LB-agar containing 20 μg ml^{-1} chloramphenicol and 200 μg ml^{-1} penicillin. A P22 phage lysate was made on a strain containing the Tn*10*dTet element and transduced into SL2741 pNK972 and selected for on LB-agar containing 20 μg ml^{-1} tetracylcine, 25 μg ml^{-1} chloramphenicol, and 50 μg ml^{-1} X-gal. Colonies that were white or dark blue were studied further by making a P22 phage lysate and transducing into a wild type *S.* Typhimurium14028s background. Transductants were then screened for cotransduction by plating on LB-agar containing 25 μg ml^{-1} chloramphenicol, LB-agar containing 20 μg ml^{-1} tetracylcine and 25 μg ml^{-1} chloramphenicol, and LB-agar containing 20 μg ml^{-1} tetracylcine. Mutants that did not show co-transduction were then checked for transposon orientation and position by using PCR with primers specific for the right and left IS elements. PCR products were then sequenced and compared against the *S.* Typhimurium LT2 genome using BLAST.

Acknowledgements

We would like to thank B. Boutt, A. Treece, K. Main, J. Wooten, and A. Nelson for their support and helpful insights. We would also like to thank H. Hassan for his help and suggestions with our anaerobic growth experiments and W. Navarre and K. Hughes for their assistance with the T-POP mutagenesis. This work was supported by grant AI148622 from the NIH to S.J.L.

Table 1. Bacterial strains and plasmids used in this study.

Strain or Plasmid	Description	Reference or source
S. enterica serovar Typhimurium		
14028s	Wild type	ATCC
SF1005	*rpoS*::pRR10 (*ΔtrfA*)	(18)
TF951	*rpoE*::*cat*	(45)
SL2686	*phoP*::*Tn10*	(19)
SL2741	P*slyA*::*lacZ* merodiploid	(6)
SL2809	*oxyR*::*Tn10*	(13)
SL2823	*soxRS*::pRR10 (*ΔtrfA*)	F. Fang
TN2958	*FNR*::*Tn10d*	(44)
SL3052	*arcA*::*Tn10*	(3)
SL3059	*dps*::pRR10 (*ΔtrfA*)	F. Fang
SL3214	*Δfur*	F. Fang
SL3343	*slyA*::*Km*	This study
SL3383	*slyA*::*Km*, pRB3::*slyA*	This study
SL3406	SL2741, pBAD::*slyA*	This study
TH1672	*fis*::*Km*	(35)
JG1148	*ihf-A::tet*	(12)
MST224	*putA*::MudJ, *relA*::*Tn10*	S. Maloy
MST1009	*ompR::Tn10*	(16)
MST3713	*hns-1*::*Km*	S. Maloy
SL3448	*STM2359::Tn10dT*	This study
Plasmids		
pBAD18	ColE1 *ori*, *araC* P_{BAD}, *rrnB*, M13	(26)
pRB3	RK2 mini-replicon, *par* stabilizing locus	(4)
pKD46	*bla* P_{BAD} *gam beta exo* pSC101 oriTS	(14)
pNK972	(P_{tac}-tnpA^{+})	(36)

Table 2. The Effect of Environmental Growth Conditions of *slyA*::*lacZ* Expression.

Growth Conditions	Fold Induction/Repression (+/- SD)
LB pH 5	1.03 +/- 0.01
LB pH 6	0.89 +/- 0.07
LB pH 9	0.98 +/- 0.02
M9 10 μM Mg^{++}	0.78 +/- 0.03
M9 1 mM Mg^{++}	1.09 +/- 0.05
MOPS 0.2% glucose	0.94 +/- 0.10
MOPS 5% glucose	1.12 +/- 0.08
LB 10 μM H_2O_2	0.92 +/- 0.04
LB 100 μM H_2O_2	0.77 +/- 0.01
LB 1 mM H_2O_2	0.61 +/- 0.04
N minimal medium	
pH 7.5 8 μM Mg^{++}	3.43 +/- 0.15
pH 7.5 200 μM Mg^{++}	1.51 +/- 0.21
pH 4.5 8 μM Mg^{++}	4.65 +/- 0.19
pH 4.5 200 μM Mg^{++}	1.67 +/- 0.22

A strain containing a chromosomal *slyA::lacZ* (SL 2741) was used for these experiments. Bacteria were grown in 10 ml LB supplemented with antibiotics as indicated and incubated at 37°C aerobically with agitation at 200 r.p.m. Cultures were inoculated with 10 μl of an overnight culture grown aerobically in 10 ml LB with antibiotics at 37°C with agitation. β-galactosidase assays were performed using a modified protocol of Miller *et* al. (31) Briefly, overnight cultures were diluted 1:10 in LB read at O.D. 600 in a spectophotometer. Three 100 μl aliquots of these dilutions were then used in the assay by diluting them in 900 μl of Z-buffer containing β-mercaptoethanol, 0.1% SDS, and chloroform to help permeabilize the cells. The cells were then vortexed for 10 seconds, placed in a 28°C water bath, and allowed to equilibrate for 10 minutes. After equilibration, 200 μl of 4 mg ml^{-1} ONPG was added and time zero was recorded. The colorometric reaction was stopped with the addition of 500 μl of 1M Na_2CO_3 and the samples were read at O.D. 420 and 550 nm in a spectrophotometer.

Table 3. *SlyA::lacZ* Expression Does Not Demonstrate Autoregulation.

Arabinose Concentration (pBAD::*slyA*)	Fold Induction/Repression (+/- SD)
0	1.00
0.01%	0.97 +/- 0.02
0.1%	1.41 +/- 0.07
0.5%	1.77 +/- 0.16
0.75%	1.80 +/- 0.08
1.0%	1.68 +/- 0.06

A strain containing a chromosomal *slyA::lacZ* was transformed with pBAD::*slyA*. These strains were grown aerobically overnight in 5 ml LB supplemented with 0.5% glucose and antibiotics as appropriate at 37ºC with agitation at 200 r.p.m. Overnight cultures were and diluted 1:10,000 in 10 ml fresh LB containing 200 μg ml^{-1} penicillin and 25 μg ml^{-1} chloramphenicol with the concentrations of arabinose shown. Bacteria were grown aerobically at 37ºC with agitation at 200 r.p.m. to an OD_{600} of 2.5. β-galactosidase assays were performed in triplicate for each sample as described previously (31). The fold induction/repression values are expressed relative to the value of the strain containing *slyA:;lacZ* and pBAD::*slyA* grown in medium without arabinose. The data shown here demonstrates that *slyA*::*lacZ* expression is not autoregulated.

Figure 1a. Survival of a *slyA* mutant in hydrogen peroxide. Bacteria were grown aerobically at 37°C in 10 ml LB supplemented with antibiotics as appropriate with agitation at 200 r.p.m. for 18 hours. Cultures were normalized and diluted to ~$3x10^7$ CFU/ml for use in the assay. A *slyA* mutant is profoundly more sensitive to hydrogen peroxide than both wild type and the complement. The survival rates represent the average for three separate experiments.

Figure 1a.

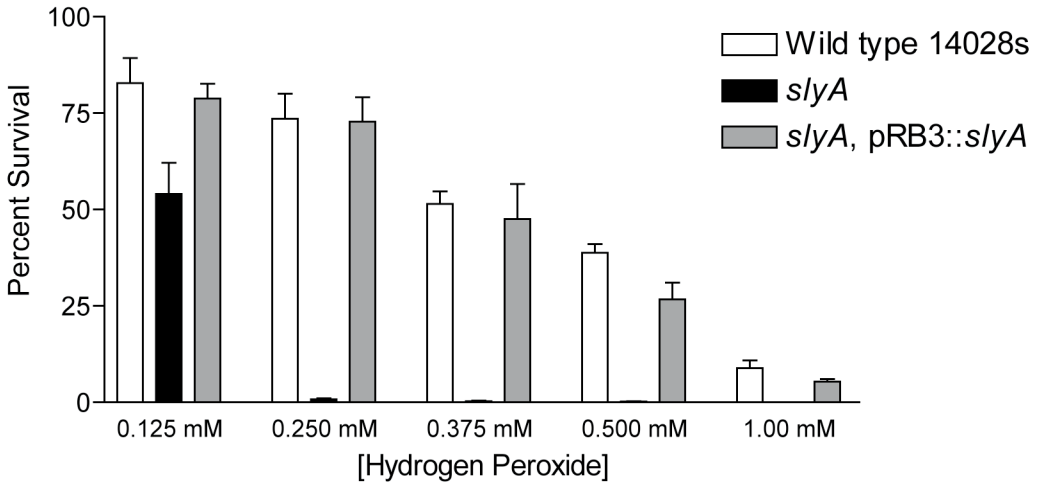

100
75
50
25
0
Percent Survival
0.125 mM
0.250 mM
0.375 mM
0.500 mM
1.00 mM
[Hydrogen Peroxide]
Wild type 14028s
slyA
slyA, pRB3::slyA

Figure 1b. Sensitivity of a *slyA* mutant to paraquat. Bacteria were grown aerobically at 37^{o}C in 10 ml LB supplemented with antibiotics as appropriate with agitation at 200 r.p.m. for 18 hours. Cultures were normalized and diluted to ~3x10^{7} CFU/ml for use in the assay. A *slyA* mutant is profoundly more sensitive to paraquat than both wild type and the complement. The diameters of sensitivity represent the average for three separate experiments with error bars indicating the standard error.

Figure 1b.

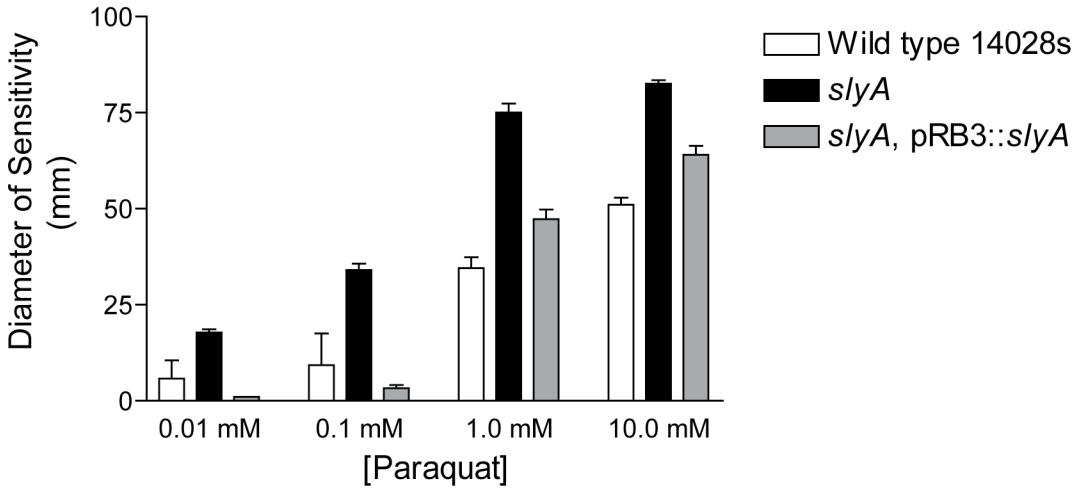

Diameter of Sensitivity (mm)
100
75
50
25
0
0.01 mM
0.1 mM
1.0 mM
10.0 mM
[Paraquat]
Wild type 14028s
slyA
slyA, pRB3::slyA

Figure 2. Aerobic vs. anaerobic growth kinetics of a *slyA* mutant. **A**. Under aerobic growth conditions, a *slyA* mutant grows considerably slower during the first five hours in culture than both wild type and a *slyA* mutant complemented by providing *slyA in trans* (0.06 $hours^{-1}$, 0.84 $hours^{-1}$, and 0.41 $hours^{-1}$). Maximum specific growth rate of the *slyA* mutant is restored, however, to that of the complement after 10 hours in culture (0.41 $hours^{-1}$ and 0.39 $hours^{-1}$ respectively). The results are the average of five independent assays with error bars to indicate standard deviations. **B**. During anaerobic growth conditions, a *slyA* mutant grows at a rate that is comparable to both wild type and the *slyA* complement (1.38 $hour^{-1}$, 1.66 $hour^{-1}$, and 1.68 $hour^{-1}$ respectively).

Figure 2a.

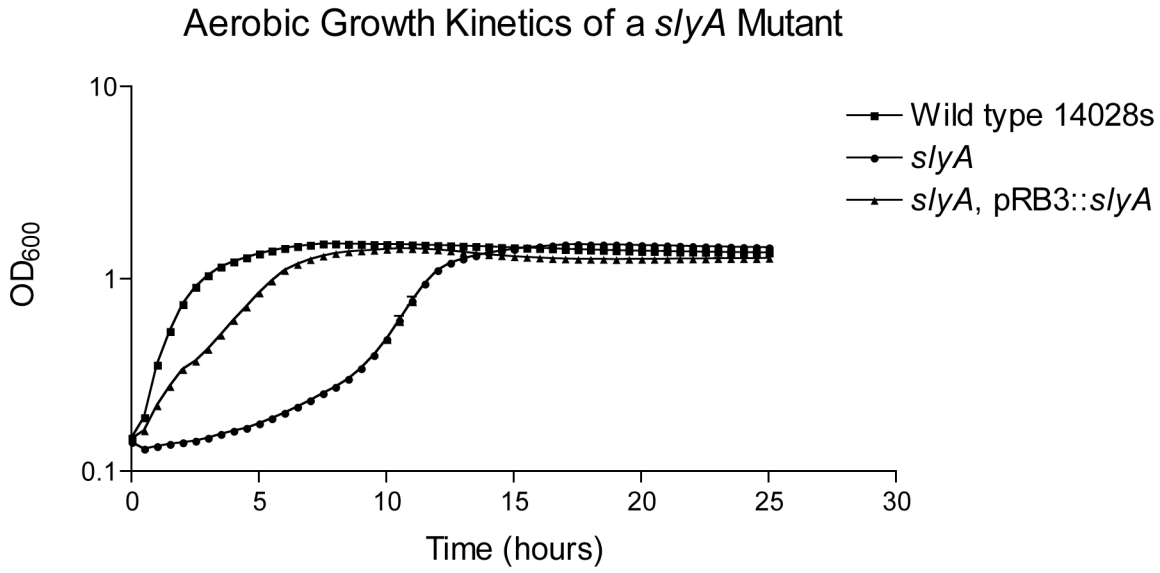


Figure 2b.

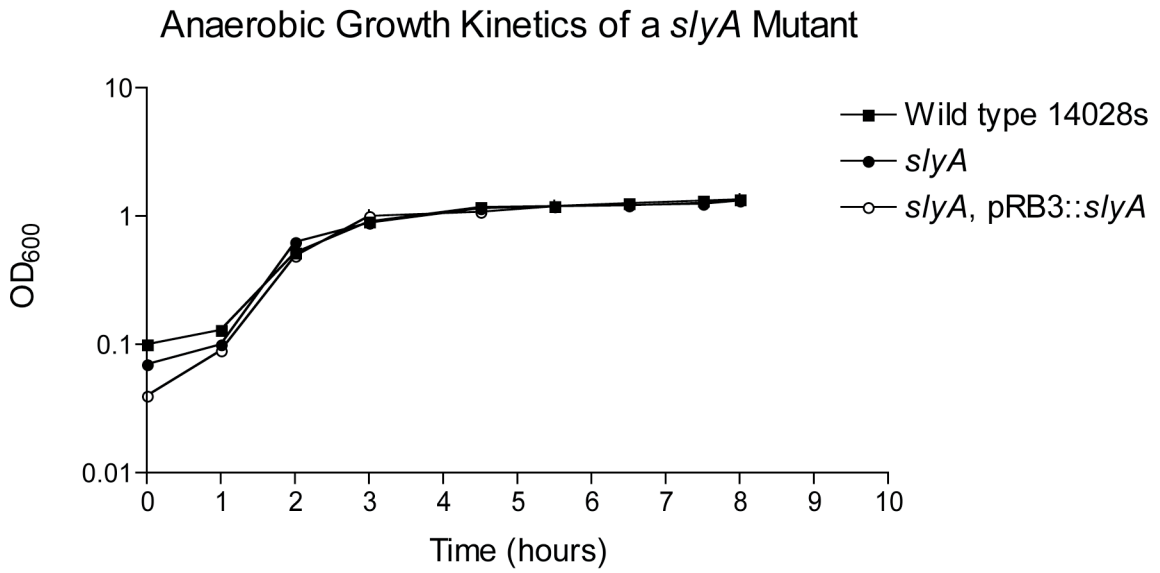


Figure 3. Aerobic survival of defined mutations in oxidative stress loci combined with a mutation in *slyA*. **A**. Aerobic survival of defined oxidative stress loci mutants. Bacteria were grown at 37°C with agitation at 200 r.p.m. Aliquots were removed daily and colony-forming units were determined by serial dilution in PBS. Enumeration of viable bacteria was done on LB agar plates incubated aerobically at 37°C. The *slyA* mutant shows profound loss of viability (< 1%) after just 24 hours in aerobic stationary-phase conditions suggesting hypersensitivity to oxidative stress. **B**. Aerobic survival of defined oxidative stress loci mutants in a *slyA* mutant background. Bacteria were grown at 37°C with agitation at 200 r.p.m. in 10 ml LB supplemented with antibiotics as appropriate. Aliquots were removed daily and colony-forming units were determined by serial dilution in PBS. Enumeration of viable bacteria was done on LB agar plates incubated aerobically at 37°C.

Figure 3a.

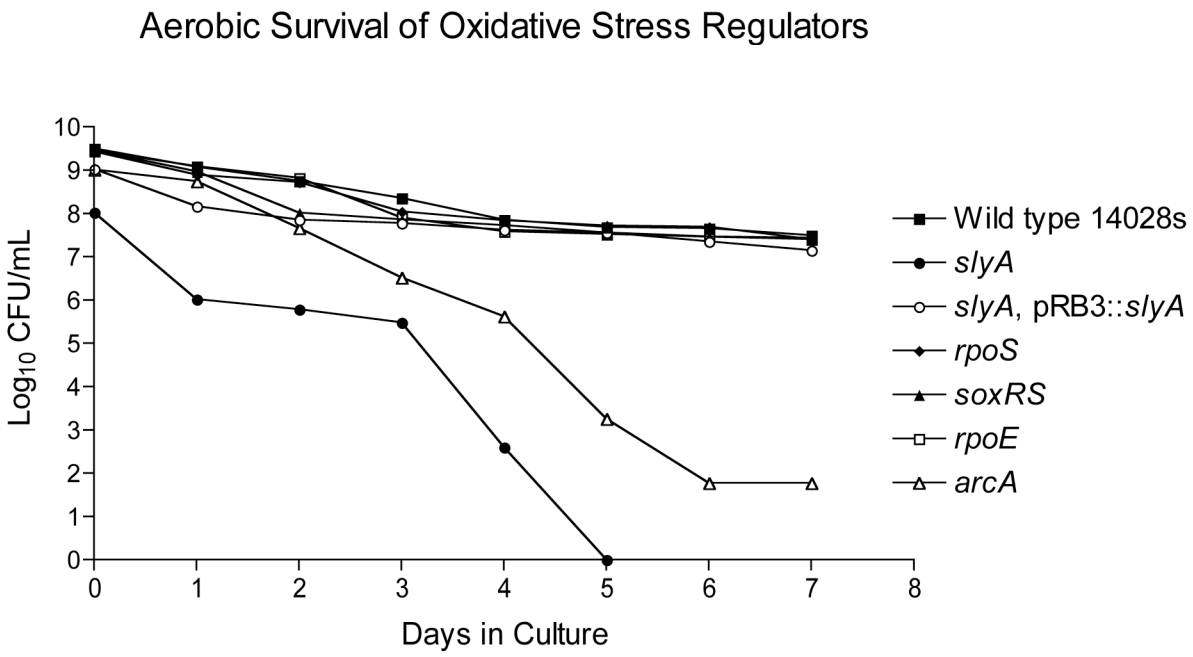


Figure 3b.

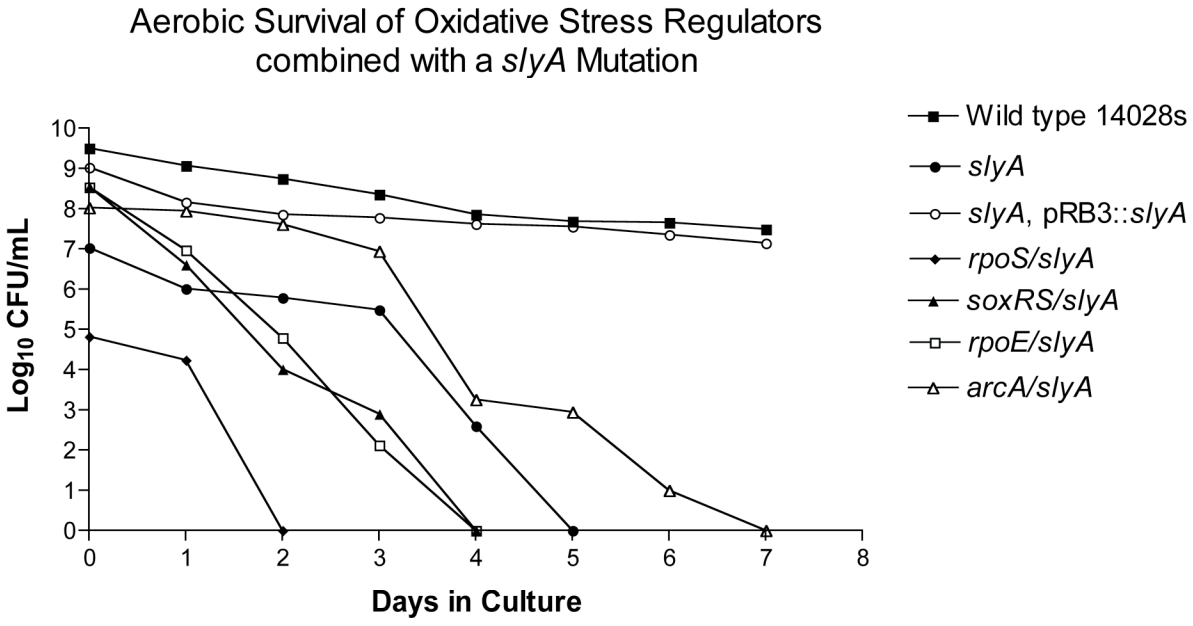


Figure 4. Anaerobic survival of defined mutations in oxidative stress loci combined with a mutation in *slyA*. **A**. Anaerobic survival of defined oxidative stress loci mutants. Bacteria were grown statically in 10 ml LB supplemented with 0.5 % glucose in an anaerobic chamber. Aliquots were removed daily, serially diluted in PBS, and plated onto LB agar to determine viable cells. **B**. Anaerobic survival of defined oxidative stress loci mutants combined with a *slyA* mutation. Bacteria were grown statically in 10 ml LB supplemented with 0.5 % glucose in an anaerobic chamber. Aliquots were removed daily, serially diluted in PBS, and plated onto LB agar to determine viable cells.

Figure 4a.

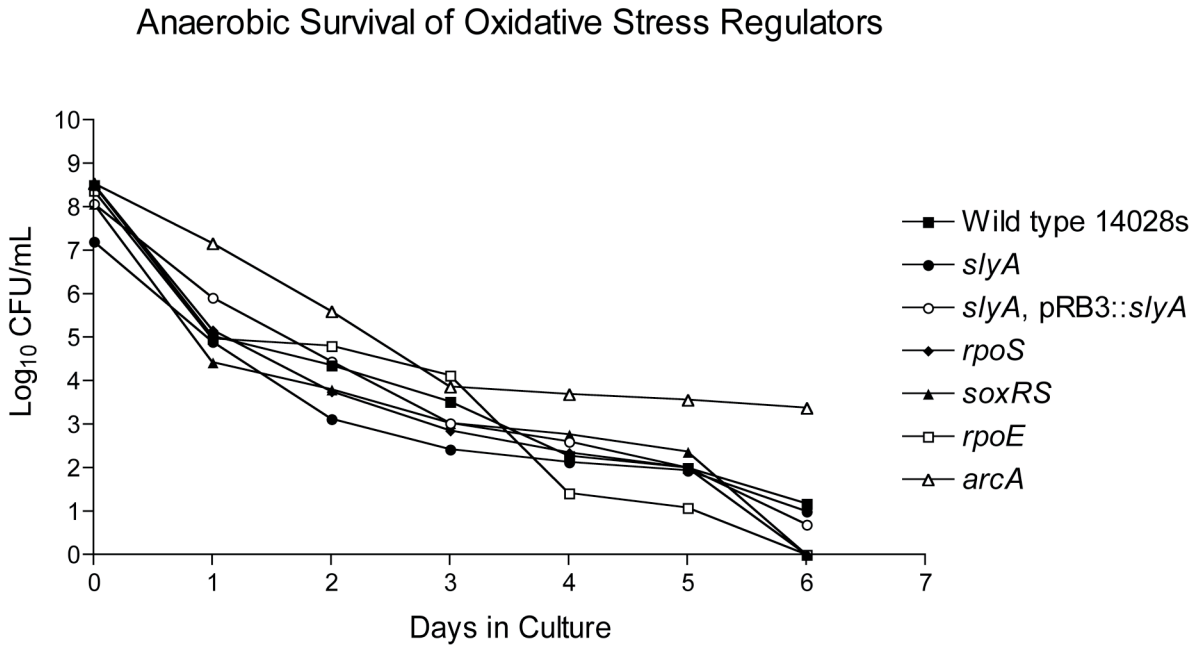


Figure 4b.

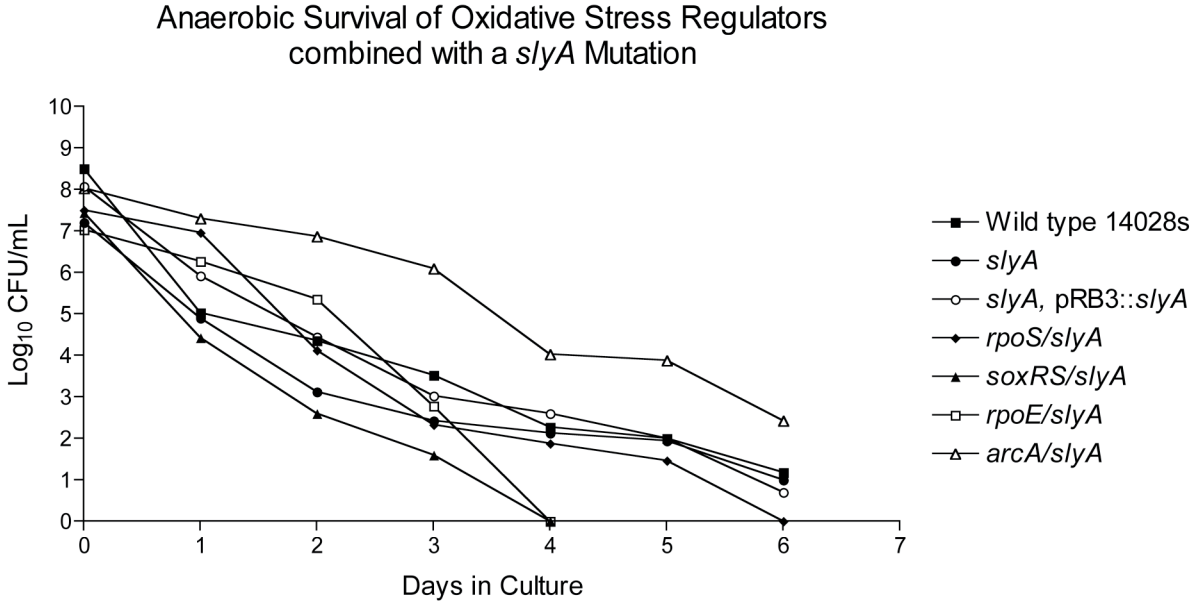


Figure 5. The influence of oxidative stress loci on *slyA::lacZ* expression. The fold induction/repression of *slyA::lacZ* expression is expressed relative to *slyA::lacZ* in the wild-type background. There is no significant effect on *slyA*::lacZ expression in mutants lacking known oxidative stress regulatory genes indicating that *slyA* functions in an independent pathway. The results are expressed as the average of three independent experiments and error bars are included to represent the standard deviations.

Figure 5.

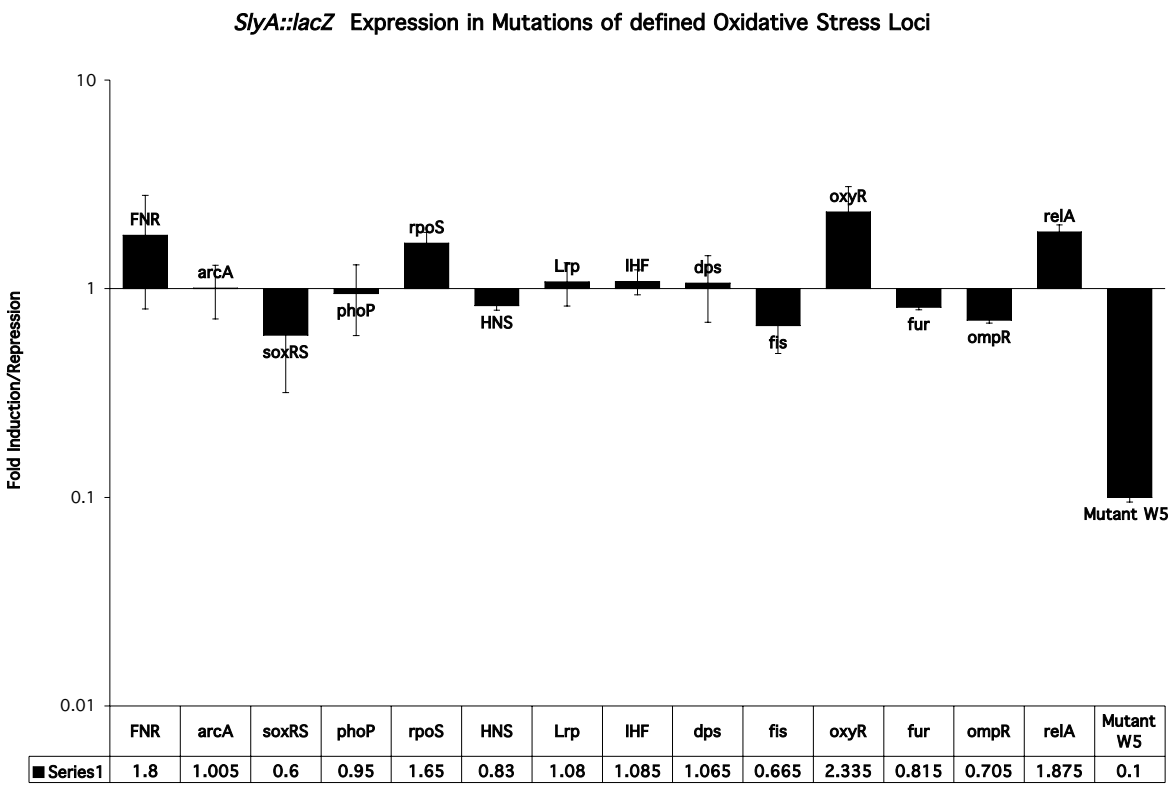

SlyA::lacZ Expression in Mutations of defined Oxidative Stress Loci
Fold Induction/Repression
10
1
0.1
0.01
FNR
arcA
soxRS
phoP
rpoS
HNS
Lrp
IHF
dps
fis
oxyR
fur
ompR
relA
Mutant W5
Series1 1.8 1.005 0.6 0.95 1.65 0.83 1.08 1.085 1.065 0.665 2.335 0.815 0.705 1.875 0.1

Figure 6. SlyA can negatively autoregulate its own expression. Overnight cultures were diluted 1:200 in fresh LB and incubated at 37^{o}C with aeration until mid-log phase. 0.2% L-arabinose was then added and the cultures were grown for another 1 hour. β-galactosidase activity was measured and results were plotted as a representation of three independent assays. Error bars are included and represent the standard deviations.

Figure 6.

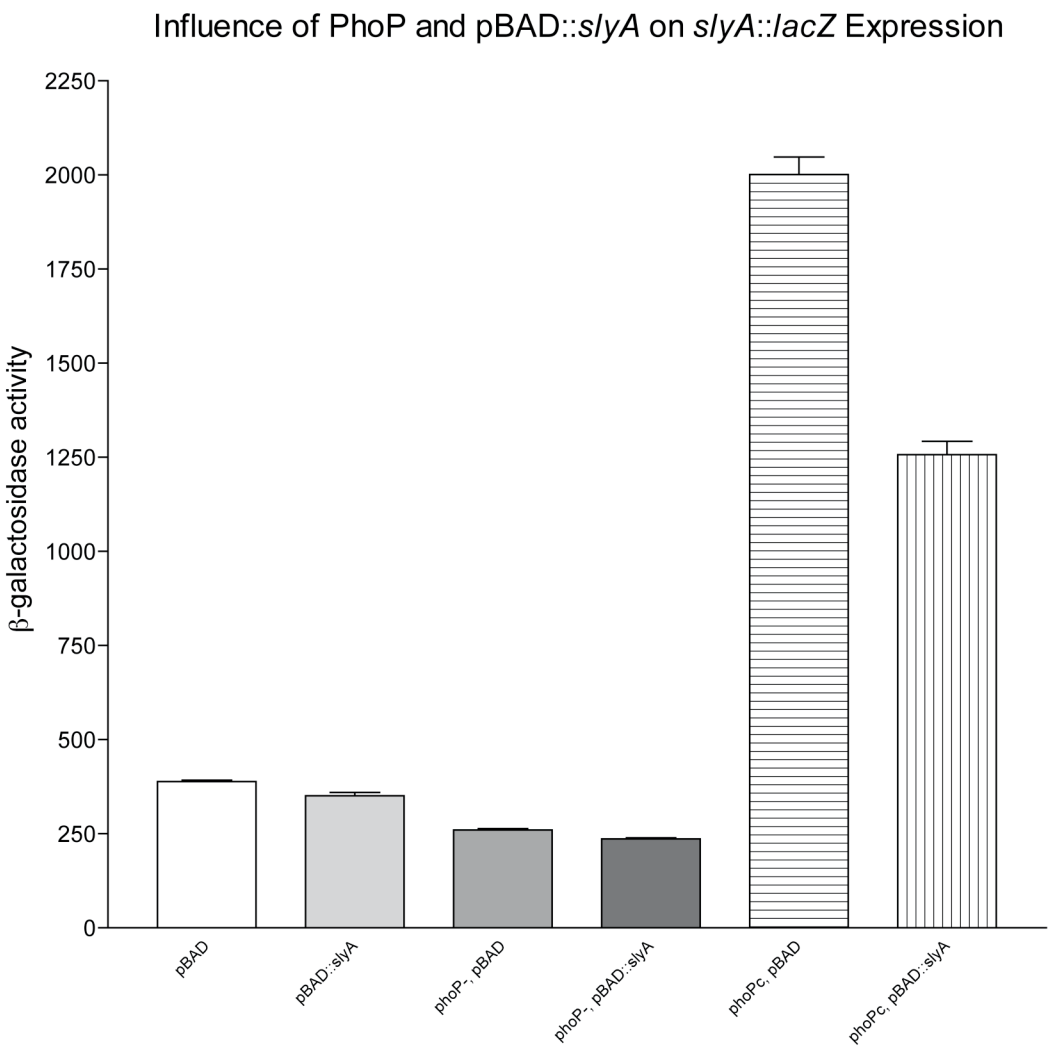

Influence of PhoP and pBAD::slyA on slyA::lacZ Expression
β-galactosidase activity
2250
2000
1750
1500
1250
1000
750
500
250
0
pBAD
pBAD::slyA
phoP-, pBAD
phoP-, pBAD::slyA
phoPc, pBAD
phoPc, pBAD::slyA

Figure 7. Quantitative real time PCR demonstrates that *slyA* expression is not significantly influenced by PhoP. Total cellular RNA was prepared from wild type *S*. Typhimurium 14028s, a *phoP* mutant, and a *phoP*-constitutive mutant grown in LB to an OD_{600} of 0.5, 1.25, and 1.75. The results are expressed as *slyA* expression relative to wild type and standardized for *gyrB* expression. The expression of *slyA* is the average expression from three independent assays with error bars included to represent standard deviations.

Figure 7.

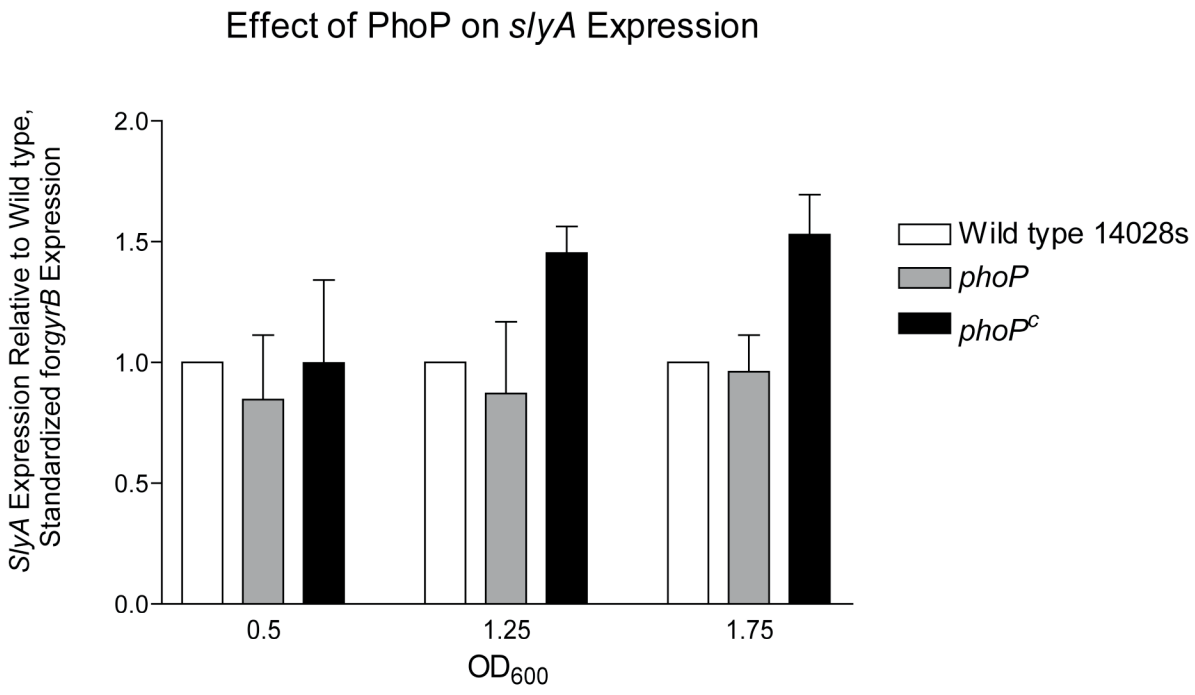
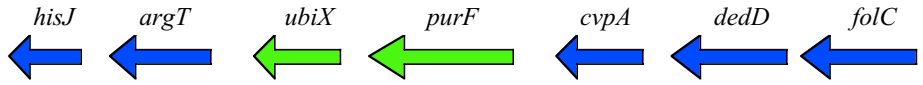

Effect of PhoP on slyA Expression
SlyA Expression Relative to Wild type, Standardized forgyrB Expression
2.0
1.5
1.0
0.5
0.0
0.5
1.25
1.75
OD600
Wild type 14028s
phoP
phoPc

Figure 8. Map of T-POP insertion. The transposon insertion into STM2359 was confirmed by PCR using arbitrary primers and primers designed to recognize the IS elements of the transposon. PCR analysis was done to amplify products both upstream and downstream of the transposon insertion. The PCR products obtained were sequenced and compared against the *S.* Typhimurium LT2 genome using BLAST.

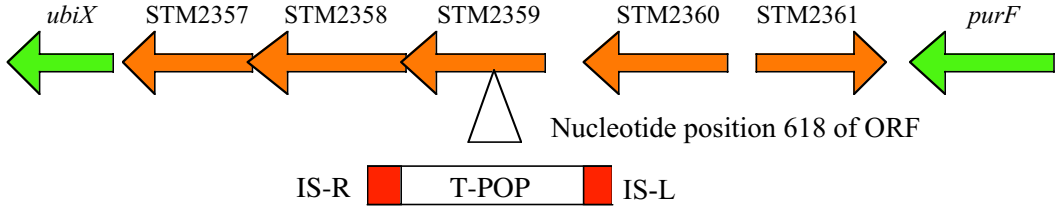
Figure 8.

E. coli ubiX to *purF* Region



Gene	Function
hisJ	histidine-binding periplasmic protein
argT	lysine-, arginine-, ornithine-binding periplasmic protein
ubiX	3-octaprenyl-4-hydroxybenzoate carboxy-lyase
purF	amidophosphoribosyltransferase
cvpA	membrane protein required for colicin V production
dedD	putative lipoprotein
folC	dihydrofolate:folypolyglutamate synthetase

S. typhimurium ubiX to *purF* Region with T-POP Insertion



Gene	Function
STM 2357	putative amino acid transporter
STM 2358	putative cytoplasmic protein
STM 2359	putative amino acid transporter
STM 2360	putative diaminopimelate decarboxylase
STM 2361	putative two-component regulatory protein

Figure 9. Hydrogen peroxide sensitivity of a transposon insertion mutant in STM2359 as compared to wild type and a *slyA* mutant. The results are expressed as percent survival and represent the average of three independent assays. Error bars are included to represent the standard deviation.

Figure 9.

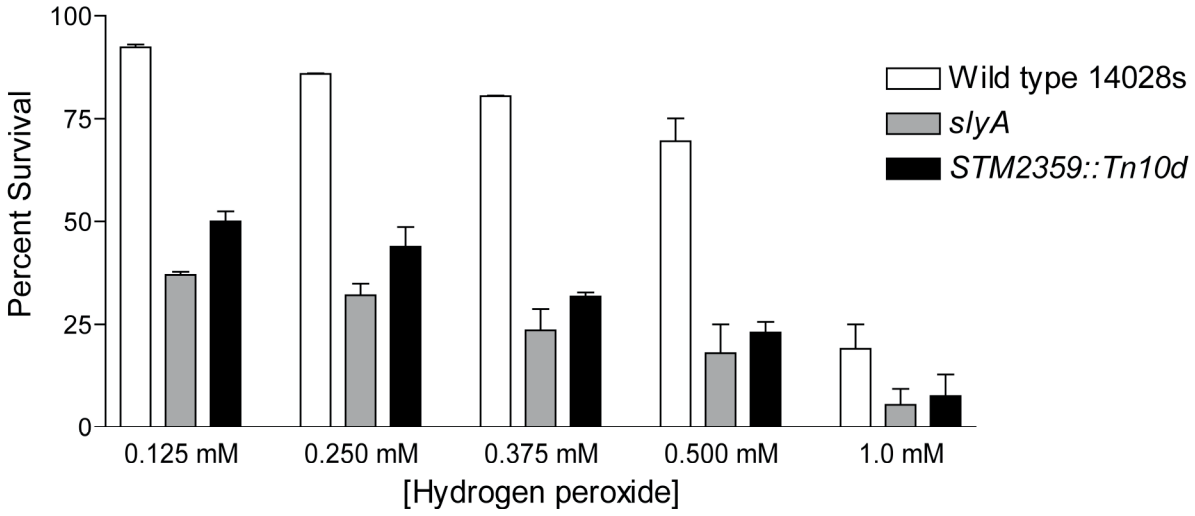
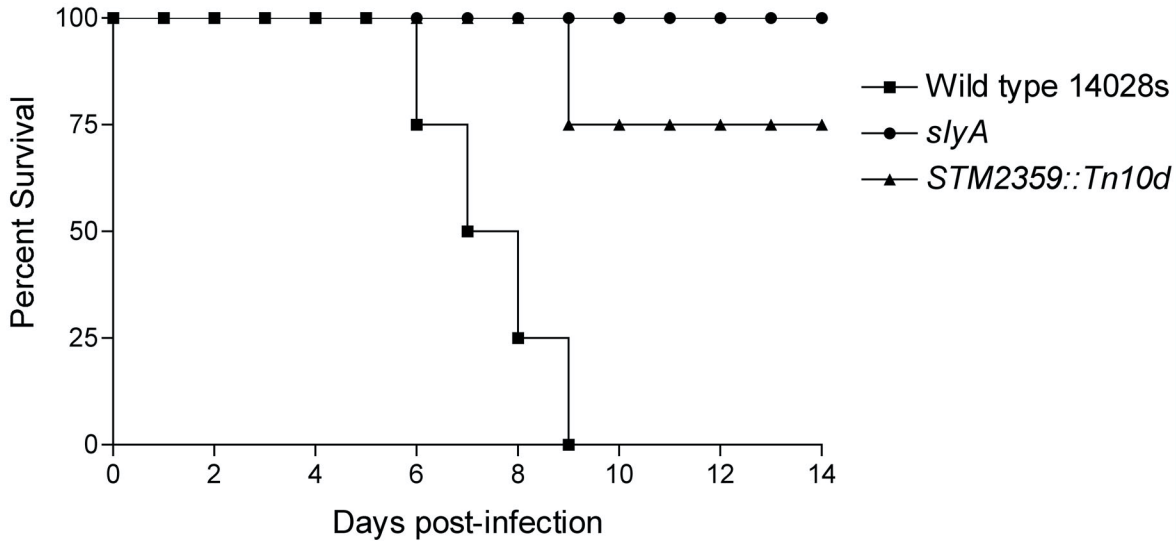

100
75
50
25
0
Percent Survival
0.125 mM
0.250 mM
0.375 mM
0.500 mM
1.0 mM
[Hydrogen peroxide]
Wild type 14028s
slyA
STM2359::Tn10d

Figure 10. The transposon insertion mutant is attenuated for virulence in C3H/HeN mice. Groups of four mice were given 1000 CFU intraperitoneally and monitored for 14 days. The results are expressed as percent survival and represent the average of two independent experiments.

Figure 10.


100
75
50
25
0
Percent Survival
0
2
4
6
8
10
12
14
Days post-infection
Wild type 14028s
slyA
STM2359::Tn10d

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Chapter 4

Unraveling the SlyA and PhoP Regulons in *Salmonella enterica* Serovar Typhimurium

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Disclaimer

This work is the result of the collaborative effort on the part of a number of laboratories across the world. Although a majority of the work presented here is a representation of my own work, there are two people who contributed the data for the DNase I protection assays. The DNase I protection assays were done by Don Walther and Linda Kenney from the Department of Molecular Microbiology and Immunology, Oregon Health and Science University, Portland, OR. Their contributions have made possible the complete story of how SlyA functions to regulate *pagC* gene expression.

Abstract

The ability to regulate gene expression is an essential aspect of bacterial survival in ever-changing environments and its pathogenesis within the host. In order to coordinately regulate gene expression, bacteria have evolved mechanisms that either activate or repress gene expression in a coordinated cellular response. These mechanisms often involve transcriptional regulatory proteins and two-component regulatory systems. The transcriptional regulatory protein, SlyA, and the two-component regulatory system, PhoP/Q are two examples of proteins involved in a coordinated gene expression when *Salmonella* resides within professional phagocytes. Since the phenotype of a *phoP/Q* mutant and *slyA* mutant *Salmonella* have similar inabilities to replicate within host macrophages, the possibility that the PhoP and SlyA regulons were intertwined was investigated. Using DNA microarray technology, we have provided evidence that the PhoP and SlyA regulons do indeed overlap. The effects of PhoP on SlyA-dependent gene expression cannot be simply explained on the basis of *slyA* transcriptional activation by PhoP. Specifically, our data demonstrates that the PhoP-activated gene, *pagC*, is indeed regulated by PhoP, however, SlyA directly induces transcription by physical association with the *pagC* promoter region. We propose that PhoP directs *pagC* transcription indirectly by directly affecting the specificity of SlyA, which binds to the *pagC* promoter and induces *pagC* gene expression. Furthermore, we demonstrate that the levels of *slyA* message do not significantly change in a *phoP* mutant or a *phoP*-constitutive mutant. Therefore, the activation of target loci by SlyA requires an activation step by either PhoP or a gene regulated by it.

Introduction

Transcriptional regulatory proteins are essential components of the complex and intricate network of gene regulation. The ability to regulate gene expression is an essential aspect of bacterial survival in ever-changing environments and its pathogenesis within the host. In order to coordinately regulate gene expression, bacteria have evolved mechanisms that can sense environmental stimuli and either activate or repress gene expression in a coordinated cellular response. These mechanisms involve two-component regulatory systems, that include a sensor kinase and a cytoplasmic regulatory protein that can act at target promoters, and these mechanisms also involve transcriptional regulatory proteins. Coordinated gene expression involving these systems is especially important in bacterial pathogenesis and virulence. The intracellular bacterial pathogen, *Salmonella enterica* Serovar Typhimurium, encodes multiple two-component regulatory systems and also several transcriptional regulatory proteins that have been found to be absolutely critical for intramacrophage survival and virulence in the murine model of salmonellosis. The transcriptional regulatory protein, SlyA, and the two-component regulatory system, PhoP/PhoQ, are two such systems that are critical for *Salmonella* pathogenesis.

The *slyA* gene was initially identified in association with hemolytic activity in *S.* Typhimurium ATCC 14028s (27, 29). The hemolytic activity was initially ascribed to SlyA itself, however, subsequent studies revealed that SlyA was responsible for the expression of a cryptic pore-forming hemolysin encoded by *clyA* or *sheA* in *E. coli* (30, 35). Subsequent research has now demonstrated that SlyA is a transcriptional regulatory protein that is required for the expression of several different genes, many of which have

yet to be identified (8, 30, 40). Many homologs of SlyA have been identified in other microorganisms suggesting that SlyA is an essential transcriptional regulatory protein. The importance of SlyA in the pathogenesis of *Salmonella* has been elucidated by several independent studies that have demonstrated that *slyA* mutants of *Salmonella enterica* Serovar Typhimurium are profoundly attenuated for virulence in the murine model of salmonellosis by the intravenous, intraperitoneal, and oral routes of infection (7, 28).

Currently, little is known about the transcriptional regulatory properties of SlyA. The observations that SlyA is essential for oxidative stress resistance, survival in macrophages, and virulence in mice suggests that there are several proteins that are either directly or indirectly regulated by SlyA that function to protect *Salmonella* from the toxic reactive oxygen species encountered within host phagocytes. The discovery of members of the SlyA regulon has been the focus of intense study in recent years and the identification of these genes will point to the role of SlyA in combating oxidative stress. The evolutionary conservation of SlyA in a very diverse group of bacteria and archea suggest that SlyA plays a more global role in microbial survival in aerobic environments and that this protein is essential for *Salmonella* pathogenesis.

PhoP/PhoQ is a two-component regulatory system that is required for virulence, is responsible for the adaptation to Mg^{2+}-limiting environments, and regulates numerous cellular activities Gram-negative bacteria. This system is encoded by the *phoP* locus, which was first identified in *Salmonella* as controlling the expression nonspecific acid phosphatase (25). The understanding of PhoP-dependent gene expression has been the focus of intense studies in recent years. A direct repeat, (T/G) GTTA has been identified in the promoter regions of the *Salmonella phoPQ* (39) and *phoN* (15) genes, however, it

has not been found in many other genes that are thought to be PhoP regulated. It is known that the PhoP-PhoQ system controls the expression of several genes that are required for growth in low Mg^{2+} concentrations (38). These include the *mgtA* and *mgtBC* genes, which encode a magnesium transport system and are encoded within *Salmonella* pathogenicity island-3 (37). Null mutations of any one of these genes cause *Salmonella* to be impaired for growth in low magnesium (38). The *ugd* and *pbgPE* –encoded proteins have been shown to be required for growth in low magnesium concentrations (38). Interestingly, they are also required for LPS modifications needed for *Salmonella* resistance to polymyxin B (13, 18). Resistance to other antimicrobial peptides, bile salts, and acid pH may also be dependent on PhoP-PhoQ as *phoP/phoQ* null mutants show increased susceptibility to these conditions (1, 10, 12, 14, 16, 19, 33, 43). Interestingly, another two-component system, the PmrA-PmrB system can also activate the loci responsible for antimicrobial peptide resistance. Activation of this system has been found to occur in the presence of high Fe^{3+} (44), however, this system can also be activated in magnesium-limiting conditions through the *pmrD* gene, which itself is regulated by PhoP (24, 26). An additional PhoP-dependent gene that is required for virulence and polymyxin resistance is *mig-14* (5). The function of this gene is unknown, however, there is some evidence that it may serve as a transcription factor. One of the first genes that was identified as a PhoP-dependent gene required for virulence was *pagC* (32). PagC is a putative membrane protein that is thought to play a role in antimicrobial peptide resistance. PagC transcription is used as a prototypical PhoP-dependent locus. The identification of other PhoP-dependent loci remains the focus of intense study and

the unraveling of the PhoP regulon will be instrumental in understanding how PhoP functions to regulate the systemic phase of *Salmonella* infection.

Although both SlyA and the PhoP/PhoQ two-component system are essential for *Salmonella* pathogenesis, little is known about the mechanisms by which the target loci function in pathogenesis. SlyA has been shown to direct the synthesis of proteins when grown within macrophages (6). PhoP/Q directs the synthesis of genes that have been shown to be actively transcribed when *Salmonella* resides within macrophages (20, 42). Since the phenotype of a *phoP/Q* mutant and *slyA* mutant *Salmonella* have similar inabilities to replicate within host macrophages, the possibility that the PhoP and SlyA regulons were intertwined was investigated. Using DNA microarray technology, we have provided evidence that the PhoP and SlyA regulons do indeed overlap. The effects of PhoP on SlyA-dependent gene expression cannot be simply explained on the basis of *slyA* transcriptional activation by PhoP (34). Specifically, our data demonstrates that the PhoP-activated gene, *pagC*, is indeed regulated by PhoP, however, SlyA directly induces transcription by physical association with the *pagC* promoter region. We propose that PhoP directs *pagC* transcription indirectly by directly affecting the specificity of SlyA, which binds to the *pagC* promoter and induces *pagC* gene expression. Furthermore, we demonstrate that the levels of *slyA* message do not significantly change in a *phoP* mutant or a *phoP*-constitutive mutant. Therefore, the activation of target loci by SlyA requires an activation step by either PhoP or a gene regulated by it.

Results

Microarray analysis of PhoP and SlyA-dependent gene expression

Studies were initiated to determine the constituents of the SlyA regulon using DNA microarrays kindly provided by Drs. Jonathan Frye and Michael McClelland at the Sidney Kimmel Cancer Center, San Diego, CA. From the list of genes that demonstrated SlyA-dependent transcription, several were previously identified as PhoP regulated loci. Three specific genes, *pagC, mig-14,* and *virK* demonstrated profound SlyA dependence as measured by DNA microarrays. To determine the specific loci that are regulated by PhoP, SlyA, and PhoP-SlyA, the transcript profiles of a *slyA* mutant, a *phoP* mutant, a constitutive *phoP* mutant and wild type *S.* Typhimurium ATCC 14028s were compared using *Salmonella* DNA microarrays. Microarray analysis of a *slyA* mutant showed significant alterations in genes that were previously reported to be PhoP-dependent as compared to wild type (2, 32). These data are summarized in Figure 1. We also compared the transcription profiles of a *phoP* constitutive mutant and a *phoP* constitutive/*slyA* double mutant. This comparison allowed for the separation of genes that were strictly PhoP-dependent and those genes that were co-regulated by both SlyA and PhoP. A *phoP* mutant was also compared to a *phoP*/*slyA* mutant to determine SlyA-dependent loci that were regulated by SlyA independent of PhoP. Collectively, microarray analysis of all of these combinations demonstrated that *pagC, mig-14*, and *virK* expression were most affected by a mutation in *slyA*. Furthermore, the microarray analysis of these mutant combinations suggest that there are specific loci that are SlyA or PhoP regulated, and these data also suggest that there specific loci that are SlyA/PhoP regulated. This is illustrated in Figure 2.

Quantitative Real-Time PCR confirms microarray data

The DNA microarray data identified loci that are SlyA and PhoP dependent. As an independent confirmation of the microarray data, we used quantitative real-time PCR to measure transcription of selected loci. The *pagC, mig-14,* and *virK* loci were chosen for RT-PCR analysis because these loci showed the greatest alterations in transcripts as determined by microarray analysis of a *slyA* mutant compared to wild type *S*. Typhimurium 14028s. Total cellular RNA was prepared from wild type, a *phoP* mutant, a *slyA* mutant, a *phoP*-constitutive mutant, a *phoP*-constitiutive/*slyA* double mutant, and a *phoP*/*slyA* double mutant grown in LB to an OD_{600} of 0.5 (log phase growth). Forty nanograms of total RNA were used in for each RT-PCR reaction. All reagents were from the Qiagen "QuantiTect SYBR Green RT-PCR,, system. The amount of product generated after each cycle was quantitated by measuring fluorescence of SYBR Green dye intercalated into double stranded DNA. Melting curve analysis verified that the reactions contained a single PCR product. Reported gene expression levels are normalized to levels of *gyrB* transcript. The expression of *gyrB* was found to be nearly identical in both control and experimental cells under these conditions. A standard curve was established by comparing *gyrB* transcript levels in serial dilutions of purified *gyrB* from the control sample.

The results of these studies demonstrate that *pagC* and *mig-14* expression are regulated by SlyA and PhoP as shown in Figure 3. A *slyA* mutant shows approximately 10-fold less *pagC* transcript as compared to wild type. Additionally, a *phoP* mutant shows approximately 35-fold less *pagC* transcript as compared to wild type. Collectively, these results suggest that PhoP may induce *pagC* expression through SlyA.

A *phoP*-constitutive mutant shows approximately 100-fold more *pagC* transcript as compared to a *phoP*-constitutive/*slyA* double mutant. These data demonstrate that SlyA is essential for *pagC* expression and that PhoP may direct this expression through SlyA. The expression of *mig-14* is more significantly affected by PhoP than by SlyA (5-fold reduced compared to 30-fold reduced). This suggestion is more clearly illustrated by the comparison of a *phoP*-constitutive mutant to a *phoP*-constitutive/*slyA* double mutant, where *mig-14* expression is only reduced approximately 2.5-fold when SlyA is absent. The expression of *virK* is not significantly affected by SlyA suggesting that *virK* expression is regulated by PhoP or an as yet to be determined mechanism.

Collectively, our data suggest that SlyA expression itself may be influenced by PhoP. In order to investigate if *slyA* expression was significantly affected by PhoP, quantitative real time PCR analysis of *slyA* was performed on wild type *S.* Typhimurium, a *phoP* mutant, and a *phoP*-constiutive mutant. The cells were grown in LB broth and total cellular RNA was prepared at early log phase, mid-log phase, and early stationary phase (OD_{600} of 0.5, 1.25, and 1.75). The results are illustrated in Figure 4. Our data demonstrate that PhoP does not significantly affect the levels of *slyA* transcript indicating that PhoP affects SlyA specificity and not *slyA* expression itself.

PagC promote fusions

In order to confirm our observations that SlyA is required for *pagC* expression, alkaline phosphatase assays were performed on a *pagC::TnPhoA* promoter fusion kindly provided by Dr. John Gunn, The Ohio State University. Mutations in *phoP* and *slyA* were also combined with the *pagC::TnPhoA* promoter fusion by P22-mediated

transduction to identify the contribution of each protein to *pagC* expression. The growth conditions used for these studies were identical to those used for microarray analysis and quantitative real time PCR. Overnight cultures were diluted 1:200 into fresh LB broth and grown to an OD_{600} of 0.5 (log phase). Alkaline phosphates assays were performed as described previously (31). Our results from this study demonstrate that both PhoP and SlyA are required for *pagC* expression as summarized in Figure 5a. Both a *phoP* and *slyA* mutant show decreased *pagC* expression (<50-fold and <55-fold respectively), and a *phoP*-*slyA* double mutant expresses approximately 100-fold less *pagC* than the wild type strain. A *phoP*-constitutive mutant, with and without a *slyA* mutation, was also used in these studies to demonstrate that SlyA is influenced by PhoP in way that permits SlyA to bind to the *pagC* promoter and induce *pagC* expression. A *phoP*-constitutive/*slyA* double mutant showed approximately 50-fold less *pagC* than did the *phoP*-constitutive mutant alone suggesting that PhoP induces *pagC* expression through SlyA. Additionally, if 10 mM $MgCl_2$ is added to the growth medium, the alkaline phosphatase activity of the promoter fusions is reduced suggesting a role of PhoP in *pagC* expression. In a *slyA* mutant, however, the *pagC* expression is not significantly different than when the cells are grown in LB alone. These data are illustrated in Figure 5b. This suggests that *pagC* expression can be induced by activated PhoP protein through a *phoP*-constitutive mutation or by growth in low magnesium conditions. The expression of *pagC*, however, is dependent on a functional SlyA. Collectively, these data confirm the differences in *pagC* expression there were obtained by quantitative real time PCR.

The SlyA protein binds to the pagC promoter

We have demonstrated that the transcription of *pagC* is dependent on SlyA. These results suggest that SlyA may be directly acting as a transcriptional regulatory protein by binding to the promoter region of *pagC*. In order to determine if SlyA physically associates with the *pagC* promoter, EMSA analysis was performed with purified SlyA protein and 200 bases of the *pagC* promoter region. Previous work in the laboratory had demonstrated that SlyA binds to the promoter region of *pagC* (4), but a much larger fragment was utilized for these studies. As illustrated in Figure 6, SlyA binds to and shifts the *pagC* promoter suggesting that SlyA is responsible for *pagC* expression. Previous studies have shown that PhoP controls the induction of *pagC* expression, however, PhoP has never been found to physically associate to the *pagC* promoter (32). To determine if PhoP could also bind to the *pagC* promoter, EMSA assays were performed with purified PhoP protein and a portion of the *pagC* promoter. The results from these studies demonstrated that PhoP does not bind to the *pagC* promoter (Personal communication with Dr. John Gunn). Taken together, our results demonstrate that SlyA is directly responsible for the induction of *pagC* expression, while PhoP may exert its effects indirectly by influencing the specificity of SlyA binding.

Additional data to demonstrate that SlyA specifically binds to the *pagC* promoter was determined by Drs. Don Walther and Linda Kenney using DNase I protection assays. The SlyA footprint of the *pagC* promoter is illustrated in Figure 7. Also shown in Figure 7 is the *pagC* regulatory region including DNase I protected region of the *pagC* promoter, the oligonucleotides that were used for EMSA analysis, and the transcription and translation start sites of *pagC*. The SlyA protected region of the *pagC* promoter

encompasses a region downstream of the transcription start site, however, upstream of the translation start site. Although this region of binding is not typical of transcriptional regulatory proteins, recent studies of other transcriptional regulatory proteins have shown that transcriptional activators can be located downstream of the transcriptional start site and still play a role in activation. For instance, in *S*. Typhimurium, OmpR has recently been shown to bind a region downstream of the *ssrB* transcription start site (9). Additionally, it has been reported that PhoP binds similarly to the *pstS* promoter of *B. subtilis* (36). Taken together, these data suggest a new mechanism by which transcriptional activators can exert their effects.

Discussion

Transcriptional regulation in bacteria involves a complex and intricate network of coordinated responses to environmental stimuli. For intracellular pathogens, such a *S.* Typhimurium, this is especially important for pathogenesis. *S*. Typhimurium has the ability to survive within the phagosomal compartment of macrophages and the bacteria are unavoidably subjected to environmental stress. In order to survive in this hostile environment, a number of transcriptional regulatory proteins are required for *S*. typhimurium pathogenesis. Among these are the transcriptional regulatory protein, SlyA, and the PhoP/PhoQ two-component regulatory system. Previous studies have demonstrated that both SlyA and PhoP are required for survival within professional phagocytes (6, 32), however, the possible connection between SlyA and PhoP has not been elucidated.

Microarray analysis comparing the genetic expression profiles of *slyA* and *phoP* mutants has enabled for the identification a possible mechanism of SlyA activation by PhoP/Q or loci regulated by it. Our data point to a possible mechanism by which PhoP/Q controls the activation state of SlyA and its ability to interact with target promoters. The further examination of selected loci by real time (quantitative) PCR, promoter fusions, and DNase I protection assays has demonstrated that both proteins are involved in their expression. Previous studies of Green *et* al. (34) suggest that PhoP regulates *slyA* expression by directly inducing *slyA* transcription. The data presented here clearly shows that there is no significant difference in the number of *slyA* transcripts present in either a *phoP* or *phoP*-constitutive mutant as compared to wild type. Furthermore, the effect of PhoP on SlyA-dependent gene expression cannot be simply explained on the basis of *slyA* transcriptional activation by PhoP.

In the case of at least certain SlyA/PhoP-dependent genes examined thus far, SlyA appears to control transcription directly, while PhoP instead appears to modulate SlyA specificity. Our model of a possible SlyA-PhoP interaction is shown in Figure 8. We propose that an activated (phosphorylated) PhoP directly interacts with SlyA and causes a change in the SlyA protein that enables it to bind to target promoters, including those of previously known PhoP-dependence. Mutations in either *slyA* or *phoP* abolish *pagC* expression, which suggests that both proteins are required for *pagC* expression. The largest difference in *pagC* expression is seen when PhoP is phosphorylated and combined with a *slyA* mutation, which demonstrates that SlyA directly controls *pagC* transcription. The DNase I protection assays and the EMSA assays also demonstrate that SlyA, but not PhoP, directly interacts with the *pagC* promoter. At least in the case of

pagC, SlyA controls transcription while PhoP controls the SlyA specificity for the promoter. Future studies are needed to dissect the PhoP/SlyA interaction and also to confirm our model that SlyA and PhoP share overlapping regulons.

Experimental Procedures

Bacterial strains and plasmids

All studies were performed using *Salmonella enterica* serovar Typhimurium ATCC 14028s or isogenic derivatives (Table 1). Genetic manipulations were performed in *Escherichia coli* DH5α (Stratagene). Bacterial strains harboring two gene mutations were made using P22 mediated transduction and made pseudolysogen-free using Evans Blue Uradine as described by Bochner (3).

Culture conditions and RNA isolation

Bacteria were grown aerobically at 37°C on Luria-Bertani (LB) agar or in LB broth supplemented with 100 μg/ml ampicillin, 50 μg/ml kanamycin, 25 μg/ml chloramphenicol, and 20 μg/ml tetracycline from Sigma as appropriate. Evans Blue Uranine medium was used to identify pseudolysogen-free transductants (3). For RNA isolation, bacteria were grown overnight at 37°C with aeration in LB broth supplemented with the appropriate antibiotics. Bacteria were then diluted 1:500 in 50 ml of LB broth and grown at 37°C with aeration until mid-log phase (OD_{600}=0.5). A 25-ml aliquot was removed and RNA metabolism was stopped by the rapid addition of 5 ml of an ice-cold mixture of ethanol:phenol (19:1 v/v). Bacteria were centrifuged at 7000 g for 10 minutes at 4°C. Culture supernatants were then removed and total RNA was isolated using the

MasterPure™ Complete RNA Purification Kit (Epicentre Technologies) as recommended by the supplier. Total RNA was also isolated at stationary phase (OD_{600}=2.5) from the same culture. Total RNA from three separate experiments was then pooled together and used for subsequent analysis.

DNA Microarrays

Salmonella DNA microarrays were kindly provided by Drs. Jonathan Frye and Michael McClelland (Sidney Kimmel Cancer Center, LaJolla, CA). Total RNA from all three separate experiments was pooled for labeling and microarray analysis. Briefly, fluorescently-labeled cDNA was synthesized from total RNA using SuperScript II reverse transcriptase (Invitrogen) primed with random hexanucleotides in a biased mixture of nucleotides (25 mM dCTP, dGTP, dATP and 10 mM dTTP) supplemented with either Cy5- or Cy3-labeled dUTP (Amersham). After labeling RNA was removed by hot-alkali treatment and labeled cDNA was cleaned using a Qiaquick PCR Purification kit (Qiagen, Valencia, CA). Equal volumes of labeled probes from the appropriate strains were then mixed with equal volume of hybridization solution consisting of 25% formamide, 5X SSC, 0.1% SDS, and 500 μg/ml salmon sperm DNA. Slides were pre-hybridized in 25% formamide, 5X SSC, 0.1% SDS, and 10 mg/ml BSA at 42°C for 45 minutes. Equal amounts of oppositely labeled cDNA were mixed together and hybridized to the *Salmonella* array. Dye switching was employed in which one array with Cy5-labeled control cDNA and Cy3-labeled mutant cDNA was compared to a second array with Cy3-labeled control cDNA and Cy5-labeled mutant cDNA. Arrays were scanned using a Packard Biosciences ScanArray 5000 and quantitated using

DigitalGENOME (Molecularware) spot finding software. Smoothed background intensity was then estimated and subtracted from the mean intensity in each spot boundary, followed by normalization by the median intensities in Cy3 and Cy5. Subsequent statistical analysis was performed by combining 6 replicates for each experiment and determining significant differential expression by using Software as described (22, 23).

Real-time (Quantitative) PCR Analysis

Expression levels of selected genes were analyzed by one-step, real-time, reverse transcriptase PCR analysis using an iCycler iQ^{TM} Real-Time Detection System (Bio-Rad, Hercules, CA). *Salmonella* RNA was prepared essentially identically to the RNA used in the microarray analysis (see above). All reagents were from the Qiagen "QuantiTect SYBR Green RT-PCR,, system. First strand cDNA synthesis and PCR was carried out according to the manufacturers instructions. Each 20 µl PCR reaction contained 10 µl SYBR Green master mix and 0.2 µl reverse transcriptase (RT mix) as indicated by the manufacturer. 7.8 µl of total *Salmonella* RNA at a concentration of 5 ng/ml was added to each reaction. Forward and reverse primers for each gene (see below) were added to a final concentration of 1 µM each in a total volume of 2 µl. The amount of product generated after each cycle was quantitated by measuring fluorescence of SYBR Green dye intercalated into double stranded DNA. Melting curve analysis verified that the reactions contained a single PCR product. Reported gene expression levels are normalized to levels of *gyrB* transcript. The expression of *gyrB* was found to be nearly identical in both control and experimental cells under these conditions. A standard curve was established by comparing *gyrB* transcript levels in serial dilutions of purified *gyrB*

from the control sample. Primers used to analyze *gyrB* levels were 5'-AACTTCCACTGACTGCCGTG (forward primer) and 5'-CAGAAAATGATTGGTC GTATGG (reverse primer).

Alkaline phosphatase assays

Alkaline phosphatase assays were performed as described previously (31). Bacteria were grown overnight at 37^{o}C with aeration in LB broth supplemented with the appropriate antibiotic. Overnight cultures were diluted 1:500 in LB broth and incubated at 37^{o}C with aeration until mid-log phase when the assays were performed. Results are expressed as arbitrary units of activity and represent the average of three independent assays with error bars that represent standard deviations.

EMSA assays

The method utilized to demonstrate SlyA binding to the *pagC* promoter region was done essentially as described in previously (4). In addition, His-tagged SlyA was purified as described in Thomas 2002 (41). Briefly, purified His-SlyA was stored at –20C° in 50% glycerol containing 10 mM Tris-HCl pH 7.6, 1mM EDTA, and 5mM DTT (Reaction-Storage buffer). Promoter fragments were isolated by PCR amplification. The 5-prime oligonucleotide was biotinolyated (MWG Biosciences, High Point, NC). PCR products were gel purified using Qiagen Gel extraction kit and quantitated by spectrophotometery. To perform the binding reactions, a 1X stock of Reaction-Storage buffer was made containing 100 μg/ml acetylated BSA. Binding assays were performed in at total volume of 15 μl containing the following: 1μl of 10 μg/ml poly[IC], 0.1-0.6 pg

DNA, 1μl of various dilutions of His-SlyA, and 1μl of competitor unlabeled DNA when required. Binding reactions were allowed to proceed for 20 minutes at room temperature, then 4 μl of Loading buffer (glycerol-bromophenol blue, xylene cyanol) added, and loaded onto a pre-run 5% acrylamide gel made in 1X TBE (BioRad Precast Gels, CA). The gels were run at 4°C, 100 volts until the bromophenol blue reached the bottom of the gel. The DNA-protein complexes were electrophoretically transferred to a nylon membrane (Biodyne B, Pall Corporation) using a semi-dry blotter at 190 mA for 30 minutes. The DNA was UV-crosslinked to the membrane. Detection of the biotin-DNA on the membrane was accomplished using the LightShift™ Chemiluminescent EMSA Kit (Pierce Biotechnology, Inc., IL) according to the manufacturer's instructions.

DNase I protection assay

DNA footprinting reactions were performed as described previously (21). The 5' *pagC* regulatory region was amplified by PCR using ^{32}P-labelled oligonucleotides. Each assay contained 3-105 c.p.m. labeled template. For the binding reaction, SlyA was incubated for 20 minutes at room temperature in a buffer containing 40 mM KCl, 4 mM Tris-HCl, pH 7.9, 1 mM EDTA, 1 mM dithiothreitol (DTT), 12% (v/v) glycerol. DNase I was added, and the reaction was stopped after 2 minutes by the addition of 20 mM EDTA, 360 mM Na acetate, pH 5.5 (final concentration). The final products were precipitated with isopropanol, washed with 70% ethanol, dried and resuspended in sequencing stop buffer. The products were separated by electrophoresis on a sequencing gel along with a sequencing ladder generated using the same primers and plasmids as templates.

Acknowledgements

We would like to thank B. Boutt and A. Treece for their support and helpful insights.

This work was supported by grant AI148622 from the NIH to S.J.L.

Table 1. Bacterial strains and plasmids used in this study.

Strain or Plasmid	Description	Reference or source
S. enterica serovar Typhimurium		
14028s	Wild type	ATCC
SL2236	*slyA::*pRR10 Δ*trfA*	(28)
SL2686	*phoP*::*Tn10*	(11)
JSG208	*phoP*^{c} (*pho24*)	(17)
JSG211	*pagC::TnPhoA* *PhoN2* *ZXX::6251 Tn10CAM*	John Gunn
SL3679	*phoP::Tn10*, *slyA::*pRR10 Δ*trfA*	
SL3684	*phoP,*^{c}	This study
	*slyA::*pRR10 Δ*trfA*	This study
SL3486	*pagC::TnPhoA* *PhoN2* *ZXX::6251 Tn10CAM* *phoP::Tn10*	This study
SL3489	*pagC::TnPhoA* *PhoN2* *ZXX::6251 Tn10CAM* *slyA::*pRR10 Δ*trfA*	This study
SL3497	*pagC::TnPhoA* *PhoN2* *ZXX::6251 Tn10CAM* *phoP*^{c} (*pho24)*	This study
SL3513	*pagC::TnPhoA* *PhoN2* *ZXX::6251 Tn10CAM* *phoP::Tn10* *slyA::*pRR10 Δ*trfA*	This study
SL3546	*pagC::TnPhoA* *PhoN2* *ZXX::6251 Tn10CAM* *phoP*^{c} (*pho24)*	This study

Figure 1. Summary of microarray analysis. Selected loci are shown to demonstrate the importance of PhoP and SlyA to their expression. The results are expressed as the $\log_{10}$ expression of wild type *S.* Typhimurium 14028s compared to a *slyA* mutant in the backgrounds of either a *phoP* or *phoP*-constitutive mutation. The genes represented in the chart are separated into four groups (A, B, C, and D). The genes in group A show the SlyA induction of these loci in a *phoP*-constitutive background. The genes shown in group B represent those loci that are SlyA repressed in a *phoP*-constitutive background. The genes in group C represent those loci that show SlyA induction in the background of a *phoP* null mutation. The genes shown in group D represent those loci that SlyA repressed in the background of a *phoP* null mutation.

Figure 1.

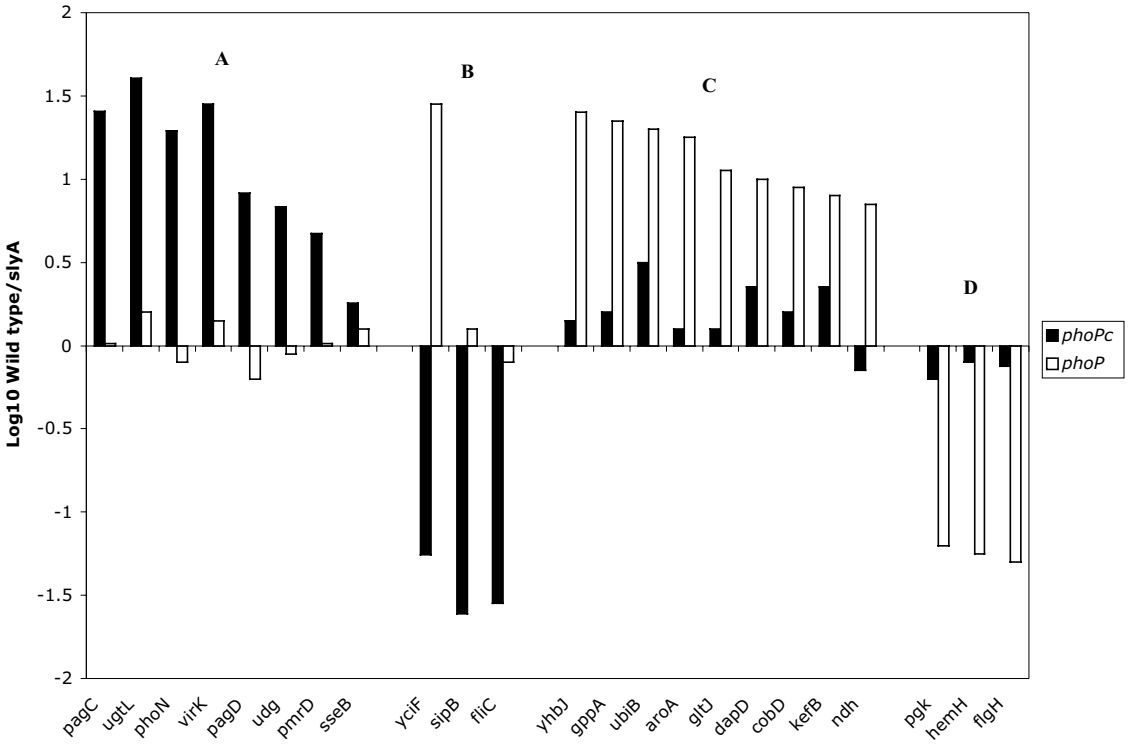

Log10 Wild type/slyA
2
1.5
1
0.5
0
-0.5
-1
-1.5
-2
A
B
C
D
phoPc
phoP
pagC
ugtL
phoN
virK
pagD
udg
pmrD
sseB
yciF
sipB
fliC
yhbJ
gppA
ubiB
aroA
gltJ
dapD
cobD
kefB
ndh
pgk
hemH
flgH

Figure 2. A pictorial representation of selected loci that are SlyA, PhoP, or SlyA/PhoP-dependent. Many of the loci listed are essential for *Salmonella* pathogenesis including resistance to antimicrobial peptides, survival in macrophages, and invasion of the intestinal epithelium.

Figure 2.

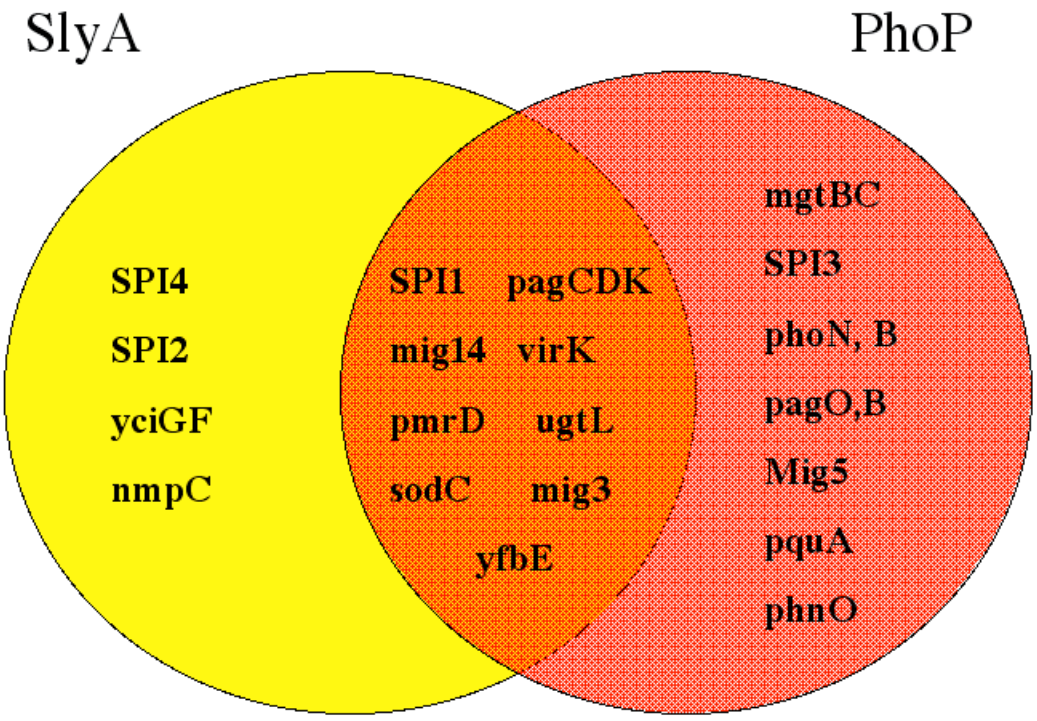

SlyA
PhoP
SPI4
SPI2
yciGF
nmpC
SPI1 pagCDK
mig14 virK
pmrD ugtL
sodC mig3
yfbE
mgtBC
SPI3
phoN, B
pagO,B
Mig5
pquA
phnO

Figure 3. Real time (Quantitative) PCR analysis of selected loci. Total cellular RNA was made from wild type *S.* Typhimurium 14028s, a *slyA* mutant, a *phoP* mutant, a *phoP*-constitutive mutant, a *phoP*-constitutive/*slyA* double mutant, and a *phoP*/*slyA* double mutant grown in LB to an OD_{600} of 0.5. The results are illustrated as expression relative to wild type and standardized for *gyrB* expression. The average of three independent assays is shown with error bars included to represent standard deviations.

Figure 3.

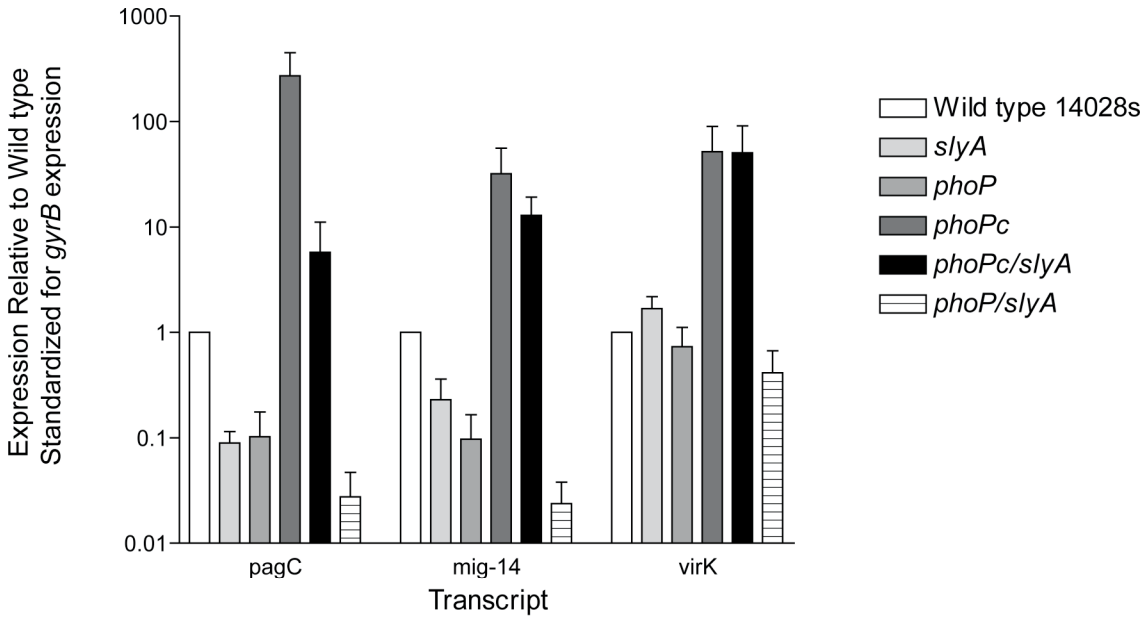

Expression Relative to Wild type
Standardized for gyrB expression
1000
100
10
1
0.1
0.01
pagC
mig-14
virK
Transcript
Wild type 14028s
slyA
phoP
phoPc
phoPc/slyA
phoP/slyA

Figure 4. Quantitative real time PCR demonstrates that *slyA* expression is not significantly affected by PhoP. Total cellular RNA was prepared from wild type *S.* Typhimurium 14028s, a *phoP* mutant, and a *phoP*-constitutive mutant grown in LB to an OD_{600} of 0.5, 1.25, and 1.75. The results are expressed as *slyA* expression relative to wild type and standardized for *gyrB* expression. The expression of *slyA* is the average expression from three independent assays with error bars included to represent standard deviations.

Figure 4.

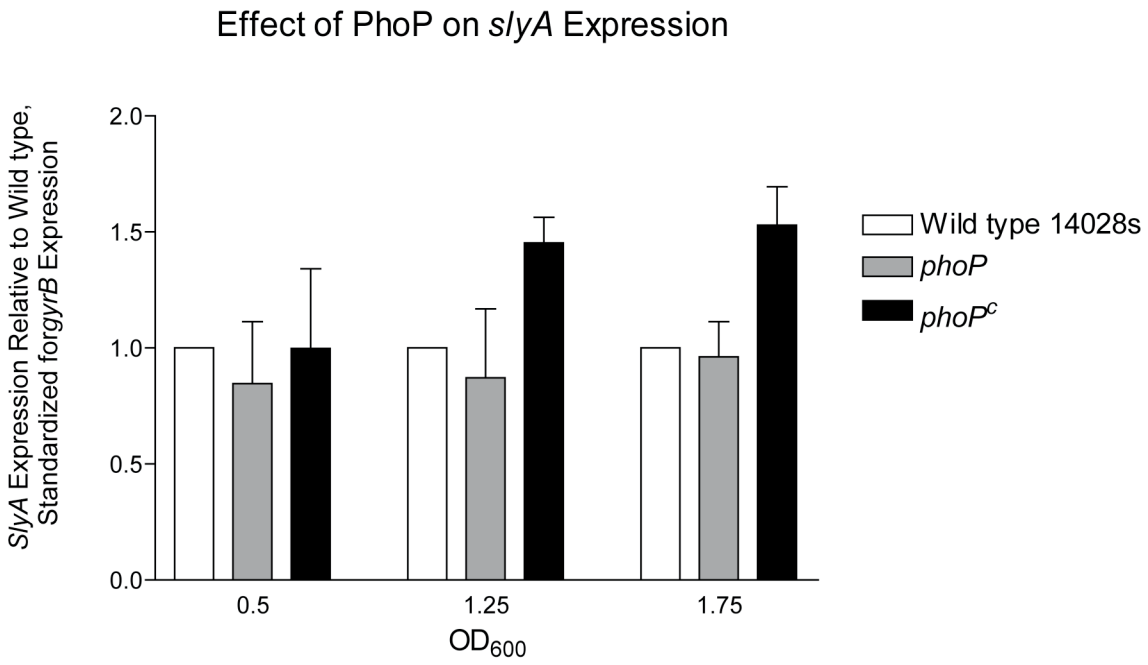
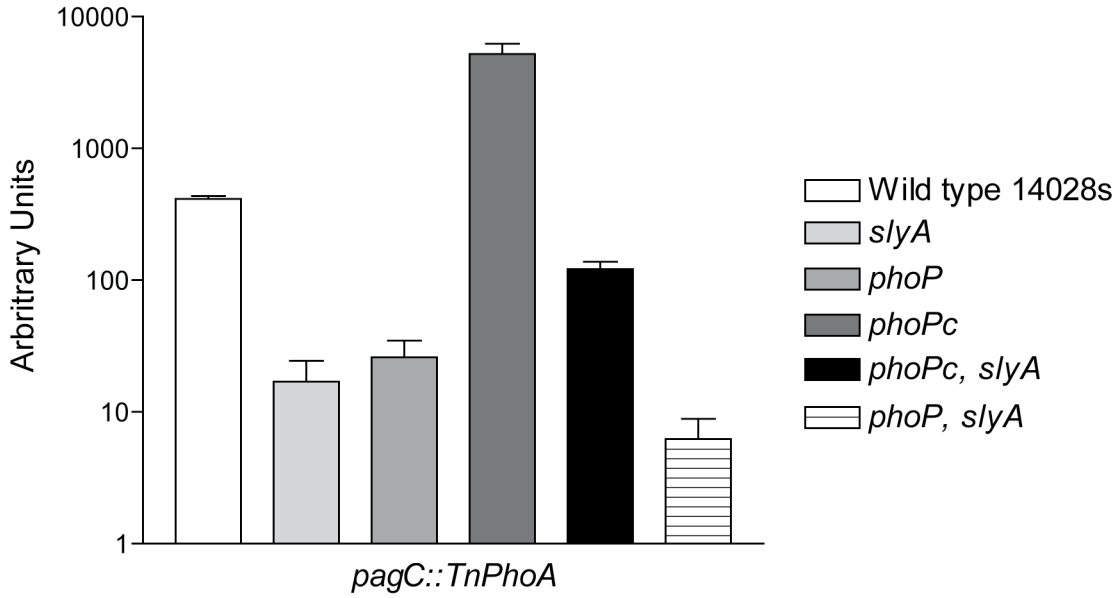

Effect of PhoP on slyA Expression
SlyA Expression Relative to Wild type, Standardized for gyrB Expression
2.0
1.5
1.0
0.5
0.0
0.5
1.25
1.75
OD600
Wild type 14028s
phoP
phoPc

Figure 5. A. *pagC* expression is regulated by both PhoP and SlyA as determined by a *pagC::TnPhoA* fusion. Genetic crosses were done using P22-mediated transduction to create double and triple mutants. Overnight cultures were diluted 1:200 into fresh LB and grown to mid-log phase when alkaline phophatase assays were performed. B. The effect of magnesium on *pagC::TnPhoA* expression. Under conditions of high magnesium ion concentration, the PhoP/Q two-component regulatory system and the loci regulated by it are not expressed. These data demonstrate that SlyA is essential for *pagC::TnPhoA* expression. The results are expressed as arbitrary units and represent the mean of three independent assays with error bars included to represent standard deviations.

Figure 5.

A.


10000
1000
100
10
1
Arbitrary Units
pagC::TnPhoA
Wild type 14028s
slyA
phoP
phoPc
phoPc, slyA
phoP, slyA

B.

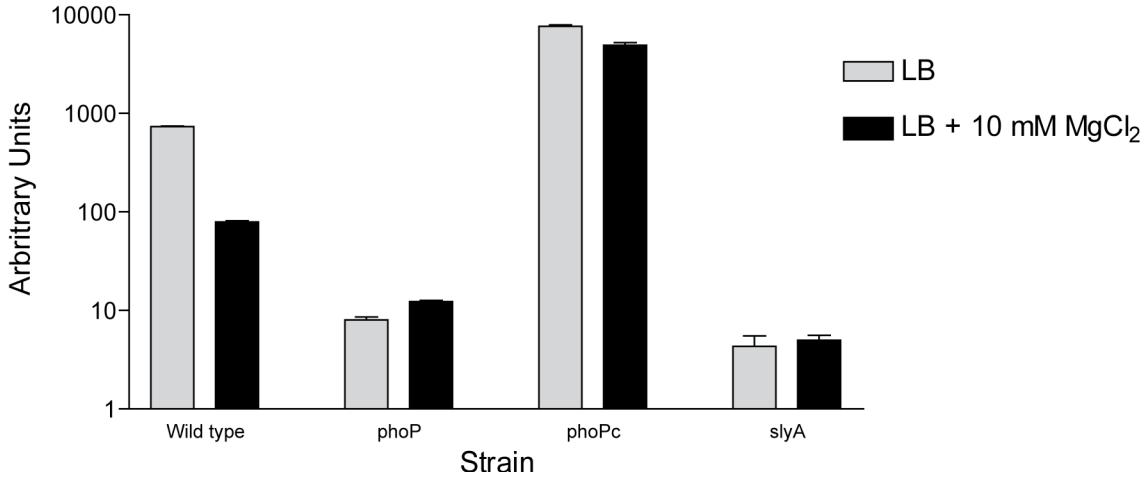

10000
1000
100
10
1
Arbitrary Units
Wild type
phoP
phoPc
slyA
Strain
LB
LB + 10 mM MgCl2

Figure 6. SlyA binds to the *pagC* promoter as determined by EMS assays. Purified His-tagged SlyA was bound to the *pagC* promoter and the complex was run on a non-denaturing polyacrylamide gel. Lanes 1-3 represent DNA-protein complexes that were incubated with increasing concentrations of unlabeled DNA. Lanes 4-12 represent labeled promoter DNA incubated with increasing amounts of purified His-tagged SlyA protein. Lane 13 is labeled promoter DNA with no protein added to the binding reaction.

Figure 6.

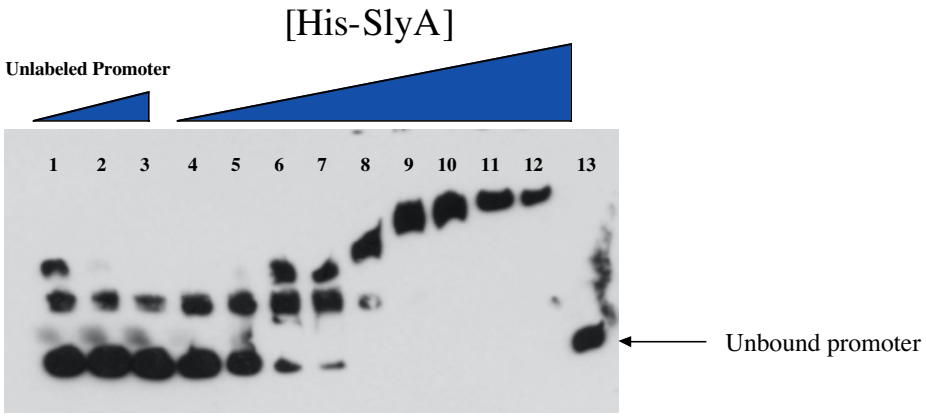
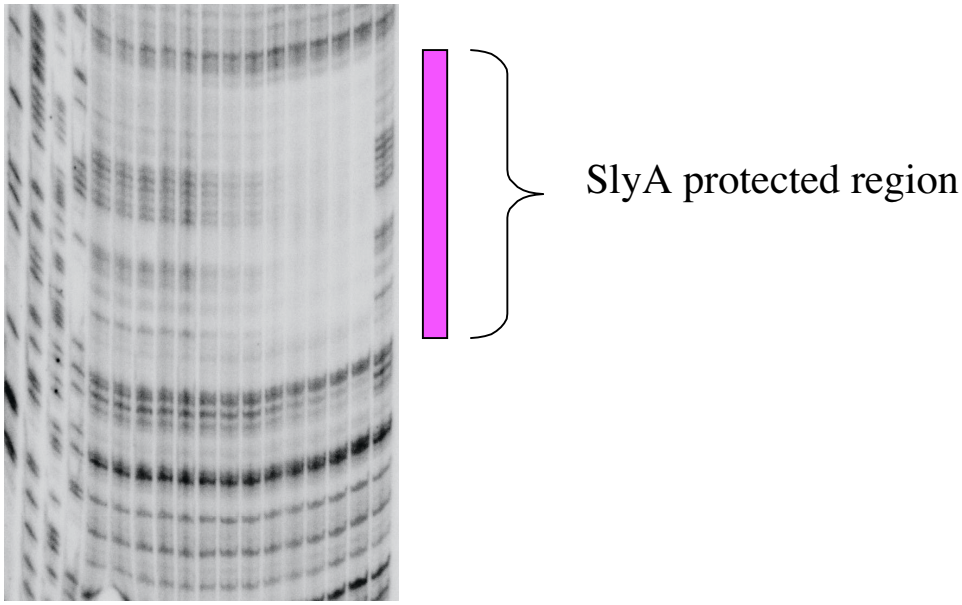

[His-SlyA]
Unlabeled Promoter
1 2 3 4 5 6 7 8 9 10 11 12 13
Unbound promoter

Figure 7. SlyA footprints the *pagC* promoter. The transcription and translation start sites as well as the SlyA protected region are shown. The oligonucleotides used for EMS analysis are also included (SL515 and SL502).

Figure 7.



pagC Promoter Region

+1

caagaaATGttgtaactgatagatatattaaaagattaaatcggagcgggaata

aagcgtgctaagcatcatcgtgaatatgattacagcgcctgcgatggcatataa

ccgtattgcggatggagcgtcacgtgaggactgtgaagcacaatgcgatatgtt

ctgattatatggcgagtttgcttaatgacatgttttagccgaacggtgtcaag

SL515

tttcttaatgtggttgtgagattttctctttaaatatcaaAATGTTGCATGGGt

gatttgttgttctatagtggctaaacACTTTATGGTTTCTGTTAAATATATATG

CGTGAGAAAAATTAGCATTCAAATCTATAAAAGTTAgatgacattgtagaaccg

SlyA protected region

gttacctaaatgagcgatagagtgcttcggtagtaaaaatatctttcaggaagt

aaacacatcaggagcgatagcggtgaattattcgtggttttgtcgattcggcat

agtggcgataactgaatgccggatcggtactgcaggtgtttaaacacaccgtaa

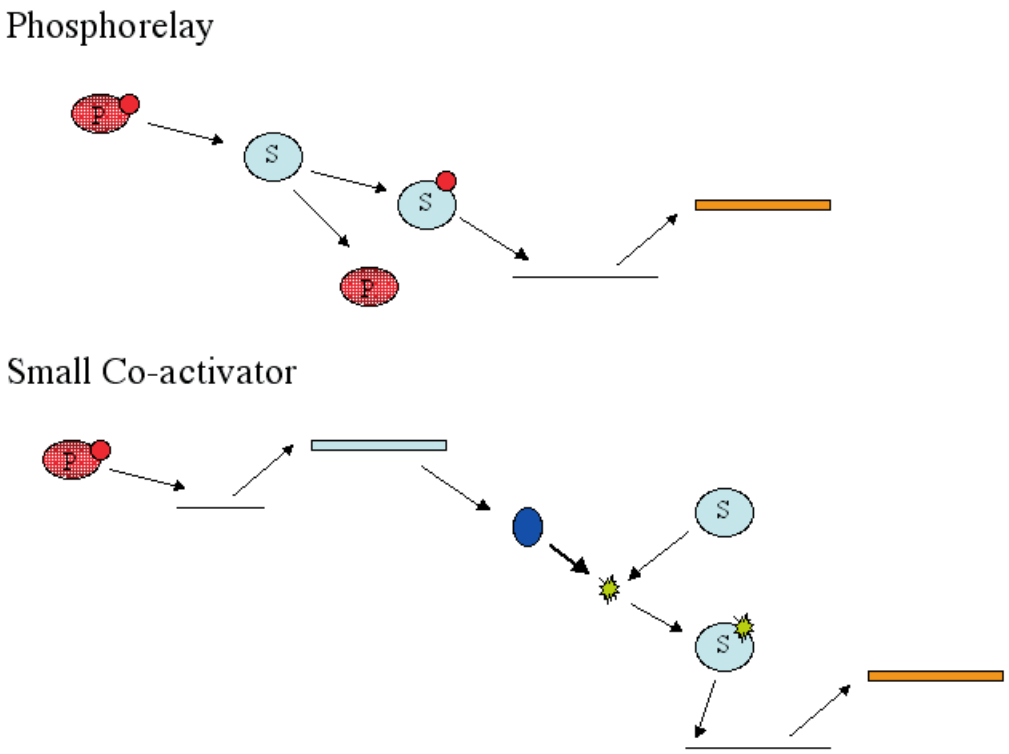
SL502

ataataaGTAGTATTAAGGAGTTGTTATGaaaaat

PagC translation start

Figure 8. Two possible models for PhoP-SlyA interaction. There are two possible models for PhoP-dependent activation of SlyA. One model is that an activated PhoP transfers a phosphate group directly to SlyA, which itself then becomes activated and can act at target promoters. A second possible model is that an activated PhoP directs the transcription of a small co-activator, which then activates SlyA.

Figure 8.



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Chapter 5

The Transcriptional Regulator, SlyA, is required for SPI2 Gene Expression in *Salmonella enterica* Serovar Typhimurium

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Disclaimer

This work is the result of the collaborative effort on the part of laboratories in England, Washington State, and our laboratory. Although a majority of the work presented here is a representation of my own work, there are a number of people who contributed the data for the competitive infection assays and the DNase I protection assays. The competitive infection studies were done by David Holden and Sheena Linehan from the Department of Infectious Diseases, Centre for Molecular Microbiology and Infection, Imperial College School of Medicine, London, UK. The DNase I protection assays were done by Don Walther and Linda Kenney from the Department of Molecular Microbiology and Immunology, Oregon Health and Science University, Portland, OR. Their contributions have made possible the complete story of how SlyA functions to regulate SPI2 gene expression.

Abstract

Salmonella pathogenicity island 2 (SPI2) is required for survival in host phagocytes and for systemic infection in mice. The SPI2 gene cluster is located at centisome 30.7 on the *Salmonella* chromosome and comprises 40 kilobases of DNA that contains 44 open reading frames, many of which encode for the two component regulatory system, SsrAB, and a type III secretion apparatus. Until recently, the genetic mechanisms of SPI2 regulation were largely unknown, however, several studies have demonstrated that the EnvZ/OmpR two-component system is required for *ssrA/ssrB* expression. In the present study, we demonstrate that SlyA is required for SPI2 gene expression through direct interaction with the *ssrB* promoter. Our results indicate that SlyA is important in SPI2 gene expression and *slyA* mutant *Salmonella* show reduced levels of SPI2 gene expression. This is the first report that demonstrates that SlyA plays a role in SPI2 gene expression and explains why *slyA Salmonella* are attenuated for virulence in mice and show reduced survival in professional phagocytes.

Introduction

The ability to regulate gene expression is an essential aspect of bacterial survival in ever-changing environments and its pathogenesis within the host. In order to coordinately regulate gene expression, bacteria have evolved mechanisms that can sense environmental stimuli and either activate or repress gene expression in a coordinated cellular response. These mechanisms involve two-component regulatory systems, that include a sensor kinase and a cytoplasmic regulatory protein that can act at target promoters, and these mechanisms also involve transcriptional regulatory proteins. Coordinated gene expression involving these systems is especially important in bacterial pathogenesis and virulence. The intracellular bacterial pathogen, *Salmonella enterica* Serovar Typhimurium, encodes multiple two-component regulatory systems and also several transcriptional regulatory proteins that have been found to be absolutely critical for intramacrophage survival and virulence in the murine model of salmonellosis.

The SsrA/SsrB two-component regulatory system is encoded within a horizontally acquired pathogenicity island, termed *Salmonella* pathogenicity island 2. SPI2 encodes a type III secretion system as well as several secreted effector proteins. Recent studies have shown that SPI2 is required for systemic infection in mice and is essential for survival in host macrophages (7, 15, 25, 28). Until recently, it was not known how the secreted effector proteins functioned to enable *Salmonella* survival within host macrophages. Elegant research by Vazquez-Torres *et al.* demonstrates that SPI-2 interferes with the trafficking of the NADPH oxidase to *Salmonella*-containing vacuoles thereby preventing phagocyte-dependent oxidative killing (33). There was a 10-fold reduction of co-localized phagosome-associated NADPH oxidase in wild type

Salmonella-infected macrophages as compared to a SPI2 mutant (33). This suggests that the secreted protein products from the SPI2 locus deflect trafficking of the vesicles containing NADPH oxidase to *Salmonella*-containing phagosomes thereby preventing *Salmonella* exposure to toxic reactive oxygen species.

The regulation of SPI2 gene expression has been the focus of intense study in recent years. Environmental stimuli, including conditions of acidic pH, have been demonstrated to be required for SPI2 gene expression, however, this was independent of the PhoP/PhoQ two-component system and Mg^{++} concentration (21). Although the PhoP/PhoQ system has been shown to be required for intramacrophage survival and virulence in mice (23), these studies suggest that SPI2 can be induced by alternative mechanisms. Recent studies of Feng *et* al. (11) have demonstrated that the OmpR/EnvZ two-component regulatory system is required for SPI2 gene expression. Collectively, these data indicate that the activation of the SPI2 locus can occur by several different and independent mechanisms.

The transcriptional regulatory protein, SlyA, has been found to be absolutely required for survival in host phagocytes and for systemic infection in mice (20). Recent studies have shown that SlyA is required for the synthesis of proteins in *Salmonella* when grown to stationary phase or within macrophages (5). The observations that SlyA is essential for oxidative stress resistance, survival in macrophages, and virulence in mice suggests that there are several proteins that are either directly or indirectly regulated by SlyA that function to protect *Salmonella* from the toxic reactive oxygen species encountered within host phagocytes. The discovery of members of the SlyA regulon has been the focus of intense study in recent years and the identification of these genes will

point to the role of SlyA in combating oxidative stress. Recent studies have demonstrated that SlyA can act as a repressor and activator of gene expression and altered levels of the proteins IroN, FliC, and PagC have been associated with a *slyA* mutation (30). These studies also demonstrated that SlyA would specifically bind to its own promoter suggesting the importance of tight SlyA regulation (30). Studies of Spory *et* al. (29) have determined that SlyA is responsible for the regulation of many proteins that are required for oxidative stress resistance. The evolutionary conservation of SlyA in a very diverse group of bacteria and archea suggest that SlyA plays a more global role in microbial survival in aerobic environments and that this protein is essential for *Salmonella* pathogenesis. Recent data of Norte *et* al (24) has determined that there is a region of the SlyA promoter that is positively regulated by PhoP and independent of the region that was found to be associated with SlyA binding (30). Furthermore, this study suggests that there may be an unknown factor through which PhoP exerts its effect (24). This finding only strengthens the idea that SlyA is an essential transcription factor required for *Salmonella* virulence and that it may play a role in gene expression with other known virulence factors. SlyA expression is also induced under conditions of low pH (5.7) and low Mg^{++} (10 μM) (our unpublished data), suggesting that SlyA plays a role in gene expression within macrophages.

The contribution of SlyA to SPI2 gene expression has yet to be elucidated. In the present study, we demonstrate that SlyA is required for SPI2 gene expression through direct interaction with the *ssrB* promoter. Our results indicate that SlyA is important in SPI2 gene expression and *slyA Salmonella* show reduced levels of SPI2 gene expression and the phenotype of a SPI2 mutant in the murine model of salmonellosis. This is the

first report that demonstrates that SlyA plays a role in SPI2 gene expression and explains why *slyA Salmonella* are attenuated for virulence in mice and show reduced survival in professional phagocytes.

Results

Microarray analysis indicates that SlyA is required for ssrB expression

In order to determine the gene expression profiles of a *slyA* mutant, total cellular RNA was isolated from wild type *Salmonella* and a *slyA* mutant grown in LB to an OD_{600} of 0.5. The RNA from wild type and a *slyA* mutant was then compared using *Salmonella typhimurium* LT2 micoarrays kindly provided by Drs. Jonathan Frye and Michael McClelland (Sidney Kimmel Cancer Center, San Diego, CA). These microarrays demonstrated that *ssrB* expression was reduced in a *slyA* mutant as compared to wild type (10-fold, Figure 1). Furthermore, microarray analysis comparing a *slyA* mutant complemented by providing *slyA in trans* and a *slyA* mutant, demonstrates that *ssrB* expression is significantly influenced by SlyA. The expression of *ssrB* in the complementing strain is similar to that of wild type. Our data demonstrate that *ssrB* expression reduced in a *slyA* mutant, thus, we would expect the genes regulated by SsrB to be reduced as well. Specifically, three of the effector proteins, *sseA*, *sseB*, and *sseC*, regulated by SsrB show reduced levels of expression in a *slyA* mutant (Figure 1). Collectively, these data demonstrate that under the growth conditions used in this study that *ssrB* expression and several of the genes regulated by it are significantly affected by SlyA.

Real time (Quantitative) PCR confirms microarray results

As an independent confirmation of our microarray data, we used quantitative real time PCR to measure *ssrB* transcription in various mutant backgrounds (Table 1). Total cellular RNA was isolated from cells grown in LB to an OD_{600} of 1.5. The expression of *ssrB* is expressed relative to wild type *ssrB* expression and standardized for *gyrB* expression. These data are summarized in Figure 2a. The expression of *ssrB* is most significantly affected in a *slyA* mutant when the cells are grown in LB (2.5-fold reduced), however, *ssrB* expression is not significantly affected in a *phoP* or *ompR* mutant (1.1-fold reduced and 1.2-fold induced respectively). The combination of mutations in *phoP*, *slyA*, and *ompR*, however, show severely reduced *ssrB* expression. An *ompR*/*slyA* double mutant has 885-fold less *ssrB* transcript as compared to wild type. In an *ompR*/*phoP* double mutant, *ssrB* expression was reduced 685-fold as compared to wild type.

To assess the contribution of growth condition on *ssrB* expression, we grew the cells in N-minimal medium at pH 5.7 containing high (10 mM) and low (10 μM) concentrations of $MgCl_2$. Briefly, the cells were grown in LB broth to an OD_{600} of 1.5. Three millileters of these cultures were centrifuged and washed two times in phosphate-buffered saline, and resuspended in a final volume of 1 mL. An aliquot of 250 μl was then added to 3 mL of N-minimal media pH 5.7 containing 10 mM $MgCl_2$ or 10 μM $MgCl_2$. These cultures were then incubated at 37°C for 2 hours and the total cellular RNA was isolated from each culture. Under conditions of low pH and low Mg^{2+} concentration, *ssrB* expression is more significantly affected by a *phoP* mutation than by a mutation in either *slyA* or o*mpR* as illustrated in Figure 2b (6-fold reduced compared to 3.1-fold and 1.2-fold reduced). As with *ssrB* expression in LB, combinations of

mutations in *phoP*, *slyA*, and *ompR* essentially abolish *ssrB* expression in conditions of low pH and low Mg^{2+}. The expression of *ssrB* under conditions of low pH and high Mg^{2+} was also investigated. Under these conditions, a mutation in *ompR* has the most significant reduction in the number of *ssrB* transcripts as shown in Figure 2c (2.4-fold as compared to wild type), however, *ssrB* expression is completely abolished in a triple mutant (*ompR, phoP,* and *slyA*). Collectively, these data demonstrate that *ssrB* expression can be significantly affected by *phoP, slyA* or *ompR* depending on the environmental growth condition. This suggests that SPI2 gene expression can be activated by several different and independent mechanisms depending on the environment.

Promoter fusions

In order to confirm our observations that SlyA is required for *ssrB* expression, β-galactosidase activity assays were performed on strains harboring a *ssrB::lacZ* reporter fusion. Mutations in *phoP*, *slyA*, and *ompR* were combined with the *ssrB::lacZ* fusion by using P22-mediated transduction. Double mutants were also made to determine the contribution of each mutation to *ssrB::lacZ* expression. Three different media conditions were also used to determine if these loci were influenced by pH or magnesium ion concentration (LB broth, N-minimal media pH 5.7, 10 μM $MgCl_2$, and N-minimal media pH 5.7, 10 mM $MgCl_2$). Although there are no clear studies demonstrating the conditions found within the phagosomal compartment of macrophages, reports by Eriksson et al. (10) suggest that the environment is acidic in pH and low in nutrients. The results of these assays are shown in Figure 3. The expression of *ssrB* is greatest under conditions

of low pH and magnesium ion concentration, which is consistent with previous reports (11). By comparing the expression of *ssrB* in all three media conditions, these data suggest that PhoP and SlyA are primarily responsible for the transcription of *ssrB*. A *slyA* mutant shows 3-fold less *ssrB::lacZ* expression when grown in LB broth, however, the effect is exaggerated when the cells are grown in low magnesium (69-fold reduced in 10 μM $MgCl_2$ and 19-fold reduced in 10 mM $MgCl_2$). There is no significant difference in *ssrB* expression in an *ompR* mutant, which is contradictory to previous reports (11). One explanation for this observation is that the OmpR binding site of the *ssrB* promoter is missing due to the method in which the *ssrB::lacZ* fusion was constructed. The expression of *ssrB::lacZ* expression is completely abolished in an *ompR*/*slyA* double mutant, which suggests that both of these regulators are important. Collectively, these data demonstrate that SlyA is required for *ssrB* expression. Under conditions of low magnesium, the effect of SlyA is enhanced suggesting that SlyA-dependent activation of *ssrB* is important within host phagocytes

SlyA binds to the ssrB promoter region

We have demonstrated that the transcription of *ssrB* is at least partially dependent on SlyA. These results suggest that SlyA may be directly acting as a transcriptional regulatory protein by binding to the promoter region. In order to determine if SlyA physically associates with the *ssrB* promoter, EMS analysis was performed with purified, His-tagged SlyA protein and 200 bases of the *ssrB* promoter region. The 200 base pair promoter fragment contains the translation start site for *ssrB* (determined by Drs. Linda Kenney and Don Walther). As illustrated in Figure 4, SlyA binds to and shifts that *ssrB*

promoter suggesting that SlyA is responsible for *ssrB* expression. Previous studies have shown that this region of the *ssrB* promoter is also bound by OmpR and by SsrB itself (11), suggesting that the transcriptional regulation of *ssrB* may be very complex.

Additional data to demonstrate that SlyA specifically binds to the *ssrB* promoter was determined by Drs. Linda Kenney and Don Walther using DNase I protection assays. In Figure 5, the *ssrB* regulatory region is shown with the DNase I protected region of the *ssrB* promoter. There are five protected regions within this promoter region (shown in red). The SlyA, SsrB, and OmpR protected regions are shown in Figure 6, as well as the transcription and translation start site for *ssrB*. Collectively, these data demonstrate that *ssrB* expression is dependent on both OmpR and SlyA and these data suggest that complete activation of *ssrB* may required both regulatory proteins.

Competitive Infection assays

The SPI2 null mutant strain used in these studies carries a non-polar disruption of *ssaV*, which is predicted to be a component of the secretion machinery (27, 28). A mutation in *ssaV* has been shown to prevent the secretion of SseB (1). A double mutant carrying mutations in *ssaV* and *slyA* was analyzed by mixed infection with either *slyA* or *ssaV* mutant strains. The competitive infection indexes obtained for comparing an *ssaV*/*slyA* double mutant to either a *slyA* or *ssaV* mutant are statistically significant from the competitive indexes of the single mutant strain relevant in each case (Figure 5). The results of these studies can only be explained if the products of these genes act dependently, and therefore SlyA regulates the expression of the SPI2 type-III secretion system genes *in vivo*.

Discussion

A coordinated cellular response is required to survival in a variety of environmental conditions. Activation of a cascade of genetic signaling involves a number of proteins that each are involved in some aspect of the pathway. Most environmental stimuli are received by two-component regulatory systems that include a sensor kinase, which receives and transmits the signal, and a response regulator, which activates downstream gene expression. The SsrA/SsrB two-component system of *S.* Typhimurium has been found to be required for *Salmonella* to cause systemic infection in mice and is essential for survival in host macrophages (7, 15, 25, 28). Elegant research by Vazquez-Torres *et al.* suggests that SPI-2 may interfere with the trafficking of the NADPH oxidase to *Salmonella*-containing vacuoles thereby preventing phagocyte-dependent oxidative killing (33). Transcriptional regulation of the SPI2 locus has recently been found to be induced by another two-component regulatory system, EnvZ/OmpR (11). This finding illustrates that a coordinated bacterial response to an environmental stimuli involves a complex and intricate network of gene expression. Furthermore, this response can involve many regulatory systems that themselves can be activated by a variety of environmental stimuli.

The work presented here demonstrates that another important transcriptional regulatory protein, SlyA, is required for *ssrB* expression and subsequent expression of the SPI2 locus. We also demonstrate that activation of the SPI2 locus can occur in the absence of either SlyA or OmpR, however, *ssrB* expression is severely reduced when an *ompR* and *slyA* mutation are combined. The competitive infection data presented here also demonstrates that SlyA directly controls SPI2 activation. The results of these studies

can only be explained if the products of these genes act dependently, and therefore SlyA regulates the expression of the SPI2 type-III secretion system genes *in vivo*. Our data also shows that activation of the SPI2 locus is greatest under conditions of low pH and low Mg^{2+}. This data suggests that the PhoP/PhoQ two-component regulatory system may be involved indirectly in *ssrB* expression, however further studies are needed to elucidate the exact mechanism of SPI2 activation by PhoP.

Experimental Procedures

Bacterial strains and plasmids

All studies were performed using *Salmonella enterica* Serovar Typhimurium ATCC 14028s or isogenic derivatives (Table 1). Genetic manipulations were performed in *Escherichia coli* DH5α (Stratagene). Bacterial strains harboring two or more gene mutations were made using P22-mediated transduction and made pseudolysogen-free using Evans Blue Uradine as described by Bochner (3).

Culture conditions and RNA isolation

Bacteria were grown aerobically at 37°C on Luria-Bertani (LB) agar or in LB broth supplemented with 100 μg/ml ampicillin, 50 μg/ml kanamycin, 25 μg/ml chloramphenicol, and 20 μg/ml tetracycline from Sigma as appropriate. Evans Blue Uranine medium was used to identify pseudolysogen-free transductants (3). For studies performed in MgM minimal medium (16), overnight cultures were grown in LB supplemented with 10 mM $MgCl_2$, then diluted 1:50 into fresh LB containing 10 mM $MgCl_2$ and incubated at 37°C with aeration until early stationary phase (OD_{600} 1.5).

Three milliliters were spun down, washed twice in phosphate buffered saline, and resuspended in 1 mL of PBS. 250 μl was then aliquoted to each of two N minimal media types containing high (10 mM) or low (10 μM) Mg^{2+} at low (5.7) pH. Cultures were then incubated at 37°C with aeration for 90 minutes and harvested. For RNA isolation, bacteria were grown overnight at 37°C with aeration in LB broth supplemented with the appropriate antibiotics. Bacteria were then diluted 1:500 in 50 ml of LB broth and grown at 37°C with aeration until mid-log phase (OD_{600}=0.5). A 25-ml aliquot was removed and RNA metabolism was stopped by the rapid addition of 5 ml of an ice-cold mixture of ethanol:phenol (19:1 v/v). Bacteria were centrifuged at 7000 g for 10 minutes at 4°C. Culture supernatants were then removed and total RNA was isolated using the MasterPure™ Complete RNA Purification Kit (Epicentre Technologies) as recommended by the supplier. Total RNA was also isolated at stationary phase (OD_{600} = 0.5) from the same culture. Total RNA from three separate experiments was then pooled together and used for subsequent analysis.

Construction of transcriptional lacZ fusions

Transcriptional lacZ fusions to the *ssrB* promoter were made using the method of Datsenko and Wanner (8). Briefly, primers 5'-CATTACTTAATATTATCTTAATTTTCGCGAGGGCAGCAAAATTCCGGGGATCC GTCGACC and 5'-CAAAATATGACCAATGCTTAATACCATCGGACGCCCCTGG GTGTAGGCTGGAGCTGCTTC were used to replace the *ssrB* open reading frame with the *aph* cassette and primers 5'-ATGATCTTCAAAAACTACAC and 5'-CTGCGTG GCGTAAGGCTCAT were used to confirm the replacement of *ssrB* with *aph*. Using the

method of Ellermeier *et* al. (9), the *aph* cassette was then resolved out using plasmid pCP20 and plasmid pCE37 was transformed into this strain creating a transcriptional *ssrB*::*lacZ* fusion.

DNA Microarrays

Salmonella DNA microarrays were provided by Drs. Jonathan Frye and Michael McClelland (Sidney Kimmel Cancer Center, San Diego, CA). Total RNA from all three separate experiments was pooled for labeling and microarray analysis. Briefly, fluorescently-labeled cDNA was synthesized from total RNA using SuperScript II reverse transcriptase (Invitrogen) primed with random hexanucleotides in a biased mixture of nucleotides (25 mM dCTP, dGTP, dATP and 10 mM dTTP) supplemented with either Cy5- or Cy3-labeled dUTP (Amersham). After labeling RNA was removed by hot-alkali treatment and labeled cDNA was cleaned using a Qiaquick PCR Purification kit (Qiagen, Valencia, CA). Equal volumes of labeled probes from the appropriate strains were then mixed with equal volume of hybridization solution consisting of 25% formamide, 5X SSC, 0.1% SDS, and 500 μg/ml salmon sperm DNA. Slides were pre-hybridized in 25% formamide, 5X SSC, 0.1% SDS, and 10 mg/ml BSA at 42^{o}C for 45 minutes. Equal amounts of oppositely labeled cDNA were mixed together and hybridized to the *Salmonella* array. Dye switching was employed in which one array with Cy5-labeled control cDNA and Cy3-labeled mutant cDNA was compared to a second array with Cy3-labeled control cDNA and Cy5-labeled mutant cDNA. Arrays were scanned using a Packard Biosciences ScanArray 5000 and quantitated using DigitalGENOME (Molecularware) spot finding software. Smoothed background

intensity was then estimated and subtracted from the mean intensity in each spot boundary, followed by normalization by the median intensities in Cy3 and Cy5. Subsequent statistical analysis was performed by combining 6 replicates for each experiment and determining significant differential expression by using Software as described (18, 19).

Real-time (Quantitative) PCR Analysis

Expression levels of selected genes were analyzed by one-step, real-time, reverse transcriptase PCR analysis using an iCycler iQ^{TM} Real-Time Detection System (Bio-Rad, Hercules, CA). *Salmonella* RNA was prepared essentially identically to the RNA used in the microarray analysis (see above). All reagents were from the Qiagen "QuantiTect SYBR Green RT-PCR,, system. First strand cDNA synthesis and PCR was carried out according to the manufacturers instructions. Each 20 µl PCR reaction contained 10 µl SYBR Green master mix and 0.2 µl reverse transcriptase (RT mix) as indicated by the manufacturer. 7.8 µl of total *Salmonella* RNA at a concentration of 5 µg/ml was added to each reaction. Forward and reverse primers for each gene (see below) were added to a final concentration of 1 µM each in a total volume of 2 µl. The amount of product generated after each cycle was quantified by measuring fluorescence of SYBR Green dye intercalated into double stranded DNA. Melting curve analysis verified that the reactions contained a single PCR product. Reported gene expression levels are normalized to levels of *gyrB* transcript. The expression of *gyrB* was found to be nearly identical in both control and experimental cells under these conditions. A standard curve was established by comparing *gyrB* transcript levels in serial dilutions of purified *gyrB* from the control

sample. Primers used to analyze *gyrB* levels were 5'-AACTTCCACTGACTGCCGTG (forward primer) and 5'-CAGAAAATGATTGGTCG TATGG (reverse primer).

β-galactosidase assays

β-galactosidase assays were performed as described previously (22, 26) as follows. Overnight cultures were diluted 1:200 in fresh LB supplemented with 10 mM Mg^{2+} and grown to an OD_{600} of 1.5 when β-galactosidase activity was calculated. Three milliliters of culture were then centrifuged at 13,000 g, washed twice in phosphate-buffered saline, and resuspended in a final volume of 1 mL. 250 μl was then added to 3 mL of N-minimal media, pH 5.7, containing either 10 μM $MgCl_2$ or 10 mM $MgCl_2$. The cultures were then incubated for 2 hours at 37°C with aeration. After incubation, β-galactosidase activity was calculated and plotted.

EMSA

The method utilized to demonstrate SlyA binding to the *ssrB* promoter region was done essentially as described in previously (4). In addition, His-tagged SlyA was purified as described in Thomas 2002 (32). Briefly, purified His-SlyA was stored at –20C° in 50% glycerol containing 10 mM Tris-HCl pH 7.6, 1mM EDTA, and 5mM DTT (Reaction-Storage buffer). Promoter fragments were isolated by PCR amplification. The 5-prime oligonucleotide was biotinolyated (MWG Biosciences, High Point, NC). PCR products were gel purified using Qiagen Gel extraction kit and quantitated by spectrophotometery. To perform the binding reactions, a 1X stock of Reaction-Storage buffer was made containing 100 μg/ml acetylated BSA. Binding assays were performed

in at total volume of 15 μl containing the following: 1μl of 10 μg/ml poly [IC], 0.1-0.6 pg DNA, 1μl of various dilutions of His-SlyA, and 1μl of competitor unlabeled DNA when required. Binding reactions were allowed to proceed for 20 minutes at room temperature, then 4 μl of Loading buffer (glycerol-bromophenol blue, xylene cyanol) added, and loaded onto a pre-run 5% acrylamide gel made in 1X TBE (BioRad Precast Gels, CA). The gels were run at 4°C, 100 volts until the bromophenol blue reached the bottom of the gel. The DNA-protein complexes were electrophoretically transferred to a nylon membrane (Biodyne B, Pall Corporation) using a semi-dry blotter at 190 mA for 30 minutes. The DNA was UV-crosslinked to the membrane. Detection of the biotin-DNA on the membrane was accomplished using the LightShift™ Chemiluminescent EMSA Kit (Pierce Biotechnology, Inc., IL) according to the manufacturer's instructions.

DNase I protection assay

DNA footprinting reactions were performed as described previously (17). Briefly, plasmids pKF6A and PKF8A were used as templates for PCR amplification of the *ssrB* 5' regulatory region. Each assay contained 3-105 c.p.m. labeled template. For the binding reaction, SlyA was incubated for 20 minutes at room temperature in a buffer containing 40 mM KCl, 4 mM Tris-HCl, pH 7.9, 1 mM EDTA, 1 mM dithiothreitol (DTT), 12% (v/v) glycerol. DNase I was added, and the reaction was stopped after 2 minutes by the addition of 20 mM EDTA, 360 mM Na acetate, pH 5.5 (final concentration). The final products were precipitated with isopropanol, washed with 70% ethanol, dried and resuspended in sequencing stop buffer. The products were separated by

electrophoresis on a sequencing gel along with a sequencing ladder generated using the same primers and plasmids as templates.

Competitive infection assays

Mouse mixed infections: Female BALB/c mice (20-25 g) were inoculated intraperitoneally (i.p.) with a 0.2 ml volume of physiological saline containing 5 x 10^5 bacteria. The cfu of each strain in the input was enumerated by plating a dilution series of the inoculum and using the appropriate antibiotic to distinguish between the strains. Mice were sacrificed 48 hours after the inoculation. The spleens were removed, placed in sterile water and homogenised by mechanical disruption. After homogenisation the samples were allowed to settle on ice for 5 minutes before transferring the supernatants into a fresh tube. Bacteria were then pelleted by centrifugation at 13.000 g and resuspended in physiological saline. Bacterial cfu were enumerated by plating a dilution series onto LB agar and LB agar with the corresponding antibiotic. The Competitive Index (CI) is defined as the ratio between the mutant and wild-type strain within the output (bacteria recovered from the spleens 48 h after i.p. inoculation), divided by their ratio within the input (initial inoculum) (13, 31). For clarity, we have re-named the CIs corresponding to mixed infections of double and single mutants strain (2, 28) Cancelled-Out Index (COI). Bacterial cfu were enumerated by plating onto LB, and LB plates containing the corresponding antibiotic. Each CI or COI value is the mean of at least three independent infections plus or minus the standard error. Student's t test was used to analyze every COI (i.e. COI corresponding to the mixed infection of strain *a* versus strain *a b)* with two null hypotheses: (1) Mean COI is significantly different from 1.0 and (2)

Mean COI is significantly different from the C.I. of the single mutant strain relevant in each case (i.e. CI of strain *b* for the case above). Probabilities (p) of 0.05 or less were considered significant.

Acknowledgements

We would like to thank A. Treece for her support and helpful insights. This work was supported by grant AI148622 from the NIH to S.J.L.

Table 1. Bacterial strains and plasmids.

Strain	Genotype	Source or reference
ATCC 14028s	Wild type *Salmonella typhimurium*	ATCC
SL2236	*slyA::*pRR10 Δ*trfA*	(20)
SL2686	*phoP::Tn10*	(12)
JSG208	*phoP^c (pho24)*	(14)
SL3630	*ompR::ahp*	This study
SL3645	*ssrB::ahp* FRT*lacZY*	This study
SL3648	*ssrB::ahp* FRT*lacZY, slyA::*pRR10 Δ*trfA*	This study
SL3652	*ssrB::ahp* FRT*lacZY, phoP::Tn10*	This study
SL3666	*ompR::ahp*, *phoP::Tn10*, *slyA::*pRR10 Δ*trfA*	This study
SL3667	*ompR::ahp, slyA::*pRR10Δ*trfA, phoP^c (pho24)*	This study
SL3668	*phoP^c (pho24), ompR::ahp*	This study
SL3676	*ssrB::ahp* FRT*lacZY, phoP::Tn10*	This study
SL3678	*ssrB::ahp* FRT*lacZY, slyA::*pRR10 Δ*trfA*	This study
SL3679	*phoP::Tn10*, *slyA::*pRR10 Δ*trfA*	This study
SL3680	*ompR::ahp, phoP::Tn10*	This study
SL3682	*ompR::ahp, slyA::*pRR10 Δ*trfA*	This study
SL3684	*slyA::*pRR10 Δ*trfA, phoP^c (pho24)*	This study
SL3685	*ompR::ahp, phoP^c (pho24)*	This study
SL3689	*ssrB::ahp* FRT*lacZY, phoP::Tn10*, *slyA::*pRR10Δ*trfA*	This study
SL3719	*ssrB::ahp* FRT*lacZY, phoP^c (pho24)*	This study
SL3720	*ssrB::ahp* FRT*lacZY, slyA::*pRR10 Δ*trfA, phoP^c (pho24)*	This study
SL3721	*ssrB::ahp* FRT*lacZY, ompR::Tn10*	This study
SL3741	*ssrB::ahp* FRT*lacZY, ompR::Tn10, slyA::*pRR10 Δ*trfA*	This study

Plasmids	Relevant characteristics	Source or reference
pKD46	*bla* P_{BAD} *gam beta exo* pSC101 oriTS	(8)
pCP20	*bla cat cI*857 λP_R *flp* pSC101 oriTS	(6)
pKD13	*bla* FRT *ahp* FRT PS1 PS4 oriR6K	(8)
pCE37	*ahp* FRT*lacZY*$^+$ t_{his} oriR6K	(9)

Figure 1. Microarray summary of selected loci of a *slyA* mutant *Salmonella*. Total cellular RNA was isolated from wild type *S*. Typhimurium 14028s and a *slyA* mutant grown in LB broth to an OD_{600} of 0.5. The results are expressed as expression relative to wild type and represent the average of two independent experiments. Error bars are included to represent standard deviations.

Figure 1.

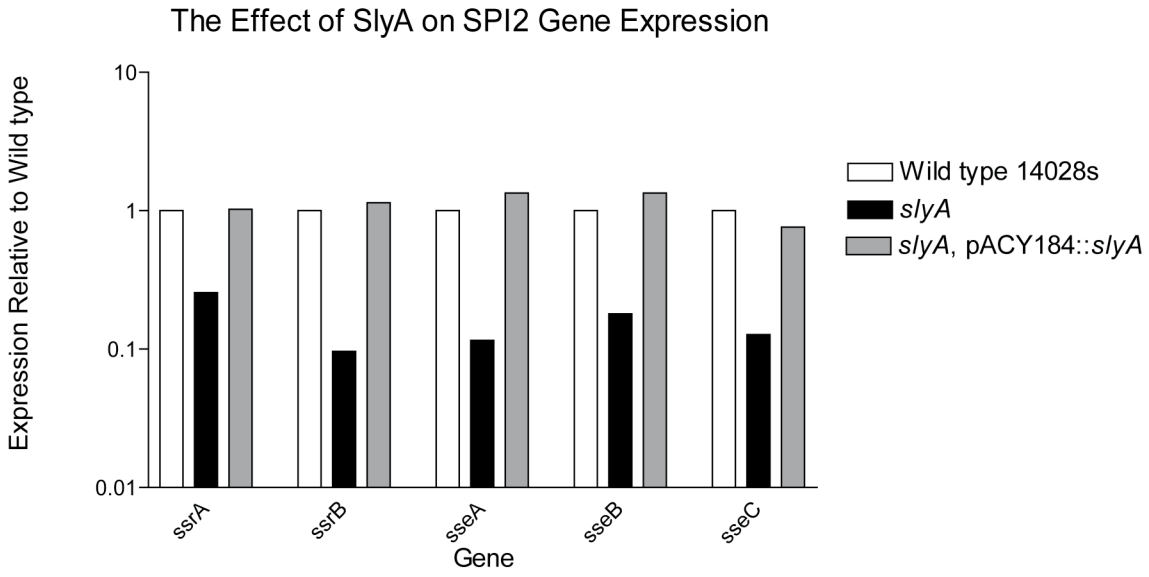
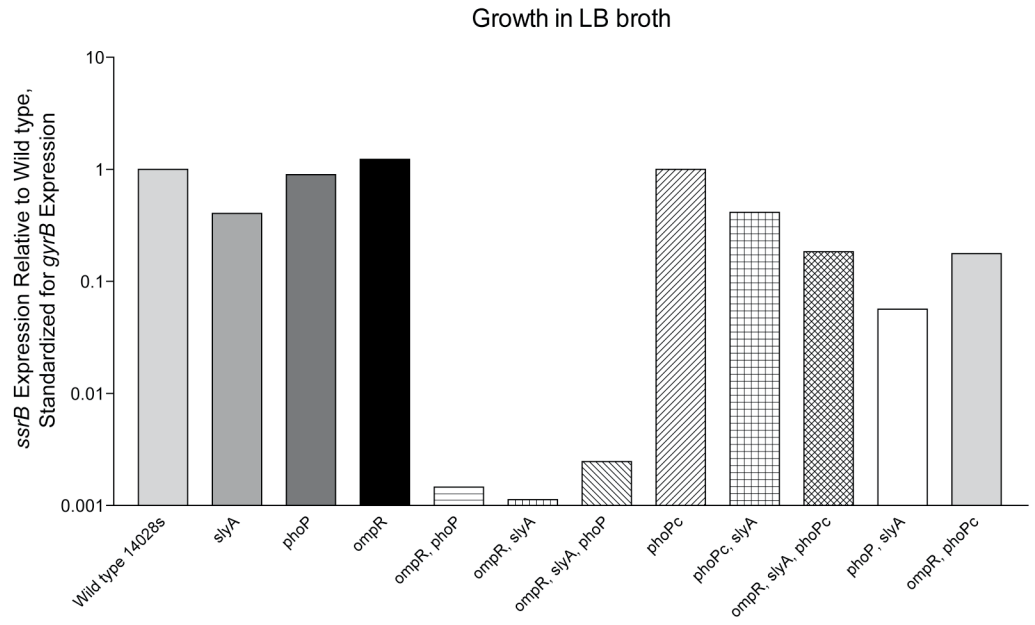

The Effect of SlyA on SPI2 Gene Expression
Expression Relative to Wild type
10
1
0.1
0.01
ssrA
ssrB
sseA
sseB
sseC
Gene
Wild type 14028s
slyA
slyA, pACY184::slyA

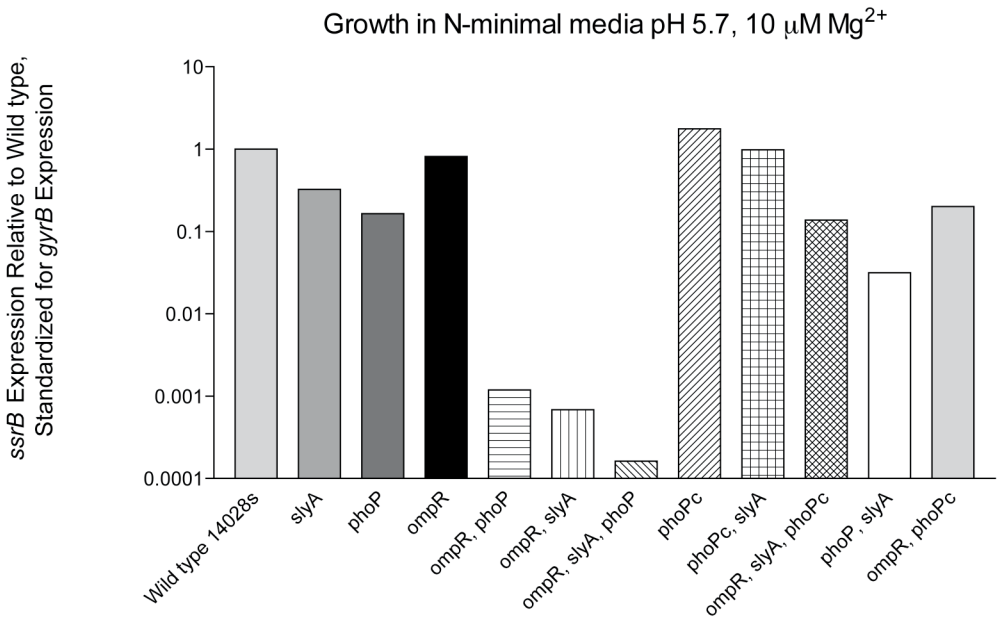
Figure 2. Real time (Quantitative) PCR analysis of *ssrB* expression in various mutant backgrounds. The cells were grown in three different media types as described in the *Materials and Methods* section. The results are presented as A. *ssrB* expression in LB. B. *ssrB* expression in N-minimal media, pH 5.7, 10 μM $MgCl_2$. or C. *ssrB* expression in N-minimal media, pH 5.7, 10 mM $MgCl_2$. The data presented here is the average of three independent assays and error bars are included to represent standard deviations.

Figure 2.

A.


Growth in LB broth
ssrB Expression Relative to Wild type, Standardized for gyrB Expression
10
1
0.1
0.01
0.001
Wild type 14028s
slyA
phoP
ompR
ompR, phoP
ompR, slyA
ompR, slyA, phoP
phoPc
phoPc, slyA
ompR, slyA, phoPc
phoP , slyA
ompR, phoPc

B.


Growth in N-minimal media pH 5.7, 10 μM Mg2+
ssrB Expression Relative to Wild type, Standardized for gyrB Expression
10
1
0.1
0.01
0.001
0.0001
Wild type 14028s
slyA
phoP
ompR
ompR, phoP
ompR, slyA
ompR, slyA, phoP
phoPc
phoPc, slyA
ompR, slyA, phoPc
phoP , slyA
ompR, phoPc

C.

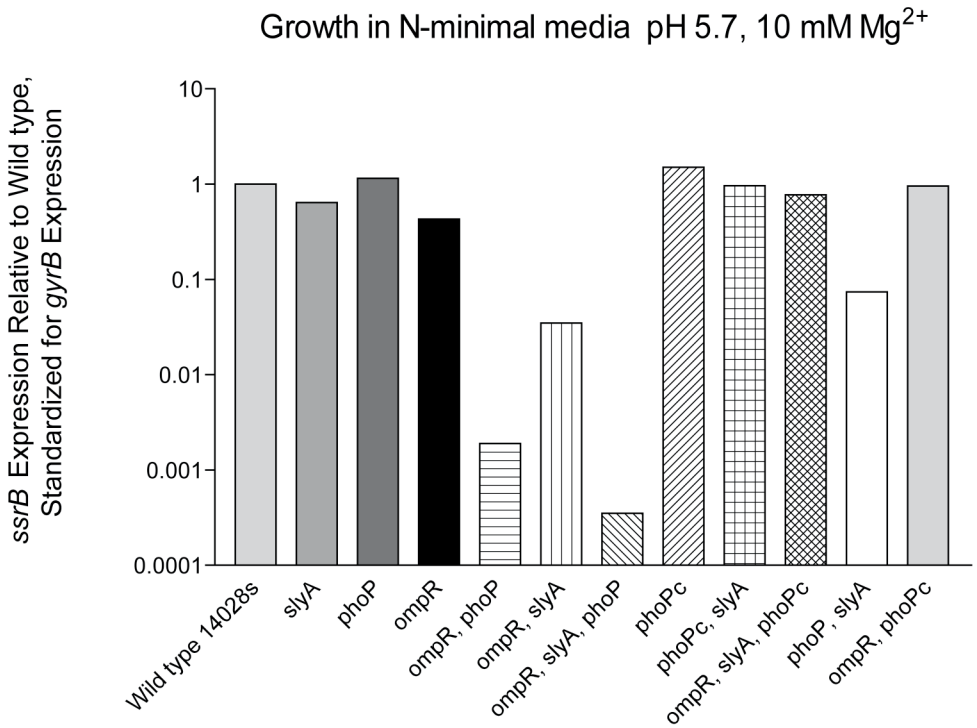
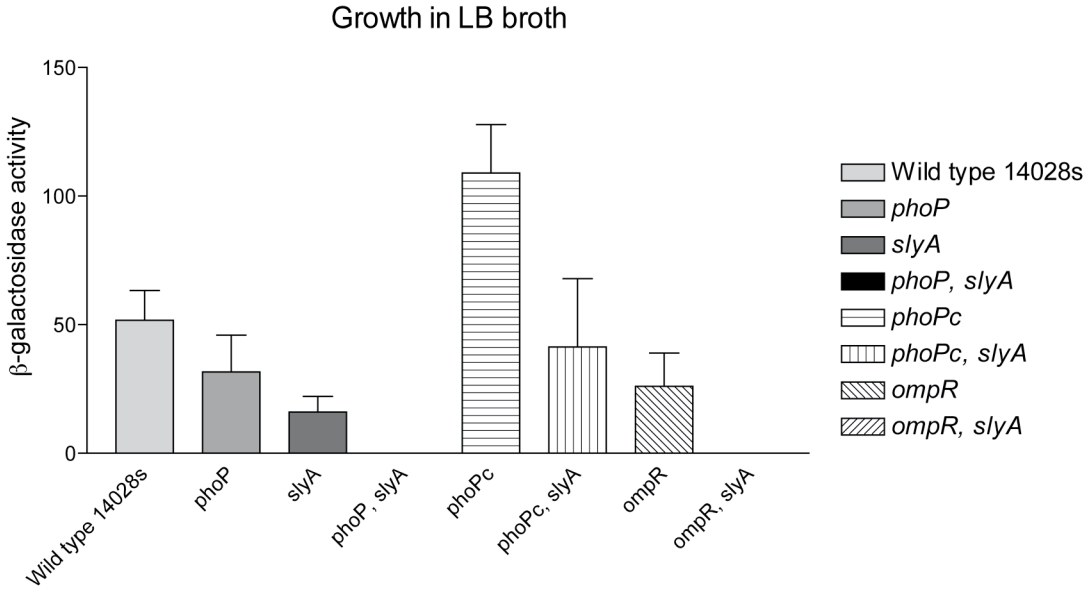

Growth in N-minimal media pH 5.7, 10 mM Mg^{2+}
ssrB Expression Relative to Wild type, Standardized for *gyrB* Expression
10
1
0.1
0.01
0.001
0.0001
Wild type 14028s
slyA
phoP
ompR
ompR, phoP
ompR, slyA
ompR, slyA, phoP
phoPc
phoPc, slyA
ompR, slyA, phoPc
phoP, slyA
ompR, phoPc

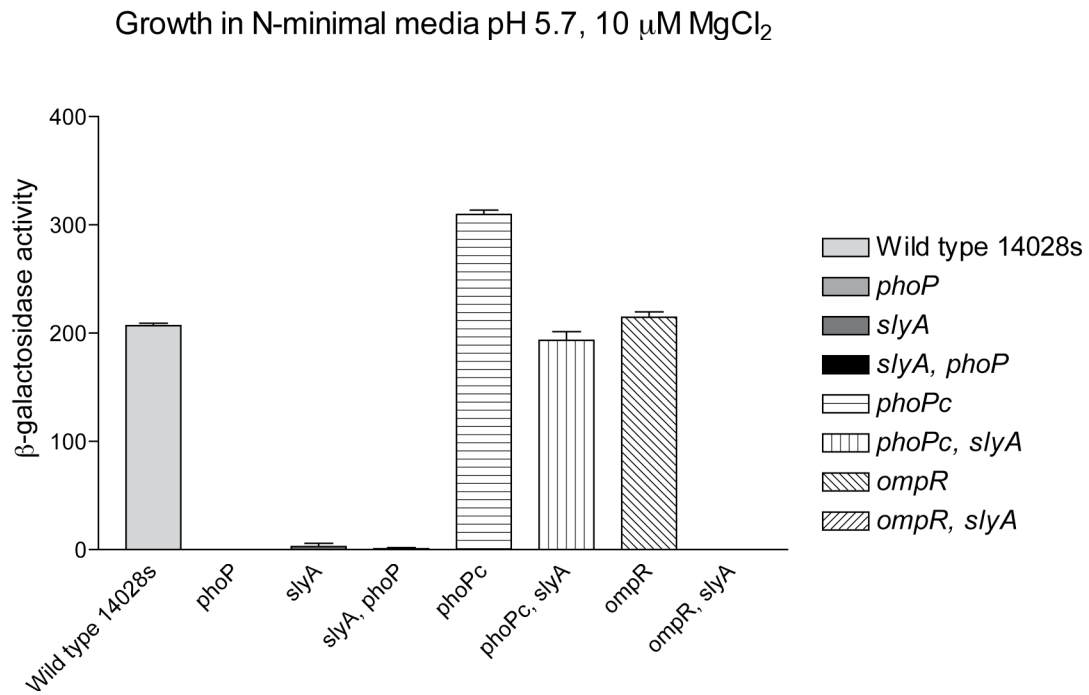
Figure 3. *ssrB::lacZ* reporter fusions demonstrate the importance of SlyA in *ssrB* expression. Strains were grown as described in the *Materials and Methods* section. The β-galactosidase activity is shown for growth in A..LB broth, B. N-minimal media, pH 5.7, 10 μM $MgCl_2$ or C. N-minimal media, pH 5.7, 10 mM $MgCl_2$. The results are expressed as β-galactosidase activity and represent the average of three assays with error bars included to represent standard deviations.

Figure 3.

A.


Growth in LB broth
β-galactosidase activity
150
100
50
0
Wild type 14028s
phoP
slyA
phoP , slyA
phoPc
phoPc, slyA
ompR
ompR, slyA
Wild type 14028s
phoP
slyA
phoP, slyA
phoPc
phoPc, slyA
ompR
ompR, slyA

B.


Growth in N-minimal media pH 5.7, 10 μM MgCl2
β-galactosidase activity
400
300
200
100
0
Wild type 14028s
phoP
slyA
slyA, phoP
phoPc
phoPc, slyA
ompR
ompR, slyA
Wild type 14028s
phoP
slyA
slyA, phoP
phoPc
phoPc, slyA
ompR
ompR, slyA

C.

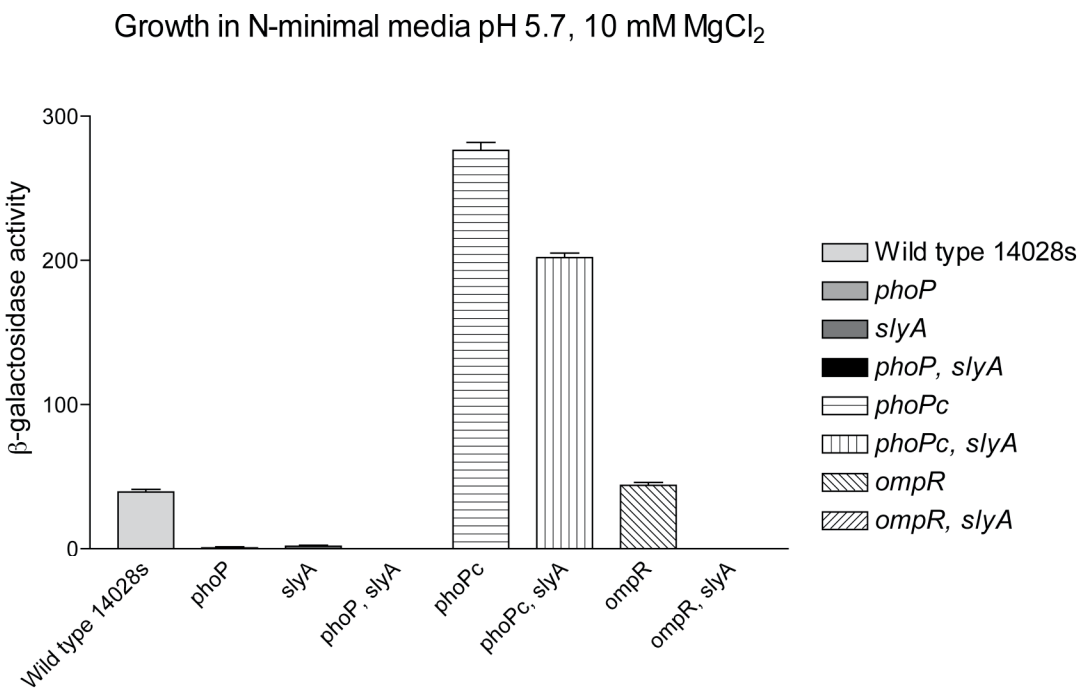

Growth in N-minimal media pH 5.7, 10 mM MgCl2
β-galactosidase activity
300
200
100
0
Wild type 14028s
phoP
slyA
phoP, slyA
phoPc
phoPc, slyA
ompR
ompR, slyA
Wild type 14028s
phoP
slyA
phoP, slyA
phoPc
phoPc, slyA
ompR
ompR, slyA

Figure 4. EMS analysis of the *ssrB* promoter with purified His-tagged SlyA protein. Increasing amounts of SlyA were incubated with a biotinylated 200 base pair fragment of the *ssrB* promoter and run on a non-denaturing polyacrylamide gel.

Figure 4.

SlyA binds to the *ssrB* promoter

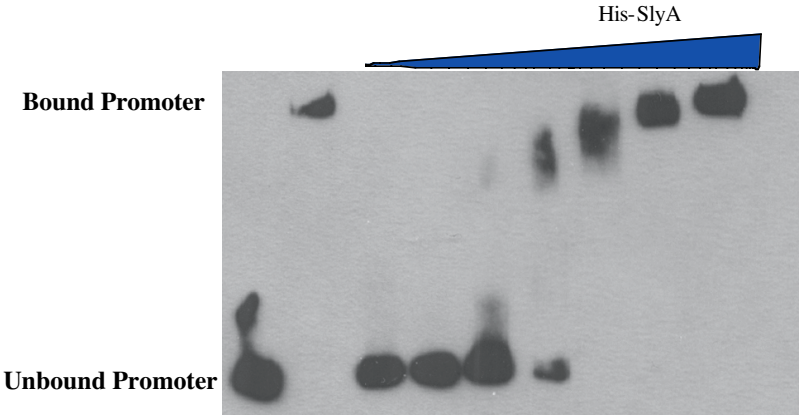
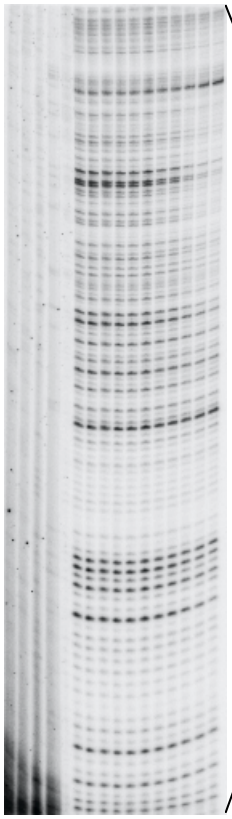


Figure 5. DNase I protection assay of *ssrB* promoter region. Increasing amounts of purified His-tagged SlyA were incubated with 200 base pair DNA fragments of the *ssrB* promoter. DNase I was added, and the reaction was stopped after 2 minutes. The products were separated by electrophoresis on a sequencing gel along with a sequencing ladder generated using the same primers and plasmids as templates. The SlyA protected region is shown in red along with the oligonucleotides (in bold) that were used for EMS analysis.

Figure 5.



DNase I Footprint of *ssrB* Promoter Region

ATTTTCCAATCACTGGACCTCTTGCTGGCTGATATTGAAAATGCCGTATCGGCTGG
AGAAAAAATCGATCAGTTAATTCACACATTAAAAGGCTGTTTAGGTCAAATAGGG
CAGACTGAATTGGTATGCTATGTCATAGACATTGAGAATCGCGTAAAAATGGGGA
AAATCATCG**CGCTGGAGGAACTAACCGAC**TTACGCCAGAA
AATACGTATGATCTTCAAAAACTACACCATTACTTAATATTATCTTAATTTTCGCGA
GGGCAGCAAAATGAAAGAATATAAGATCTTA**TTAGTAGACGATCA**
TGAAATCATCA**TTAACGGCATTATGAATGCCT**
TATTACCCTGG**CCTCATT**T**TAAAAT**TG**TAGAGCA**
TGTTAAAAATGGTCTTGAGGTTTATAATGCCTGTTGT**GCATACG**
AGCCTGACATACTTATCCTTGATCTTAGTCTACCTGGCATCAAT
GGCCTGGATATCATTCCTCAATTACATCAGCGTTGGCCAGCAATGAATATTCTGGT
TTACACAGCATACCAACAAGAGTATATGACCATTAAAACTTTAGCCGCAGGTGCTA
ATGGCTATG

BLACK = primers used for *ssrB* gel-shift

RED = Dnase I protected region

Figure 6. The *ssrB* regulatory region. The SlyA, OmpR, and SsrB protected regions are shown. The 200 base pair promoter fragment that was used for EMS analysis includes the SlyA protected region and portions of the OmpR and SsrB protected regions. The SlyA protected region is downstream of the *ssrB* transcription start site, however, it is upstream of the *ssrB* translation start site.

Figure 6.

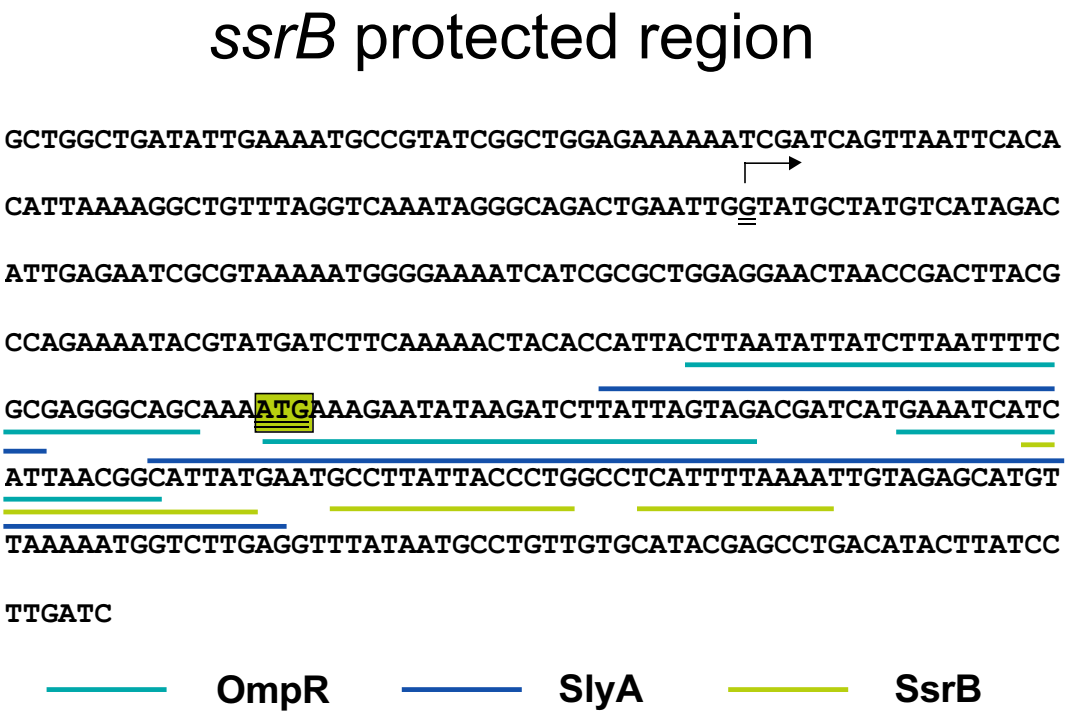
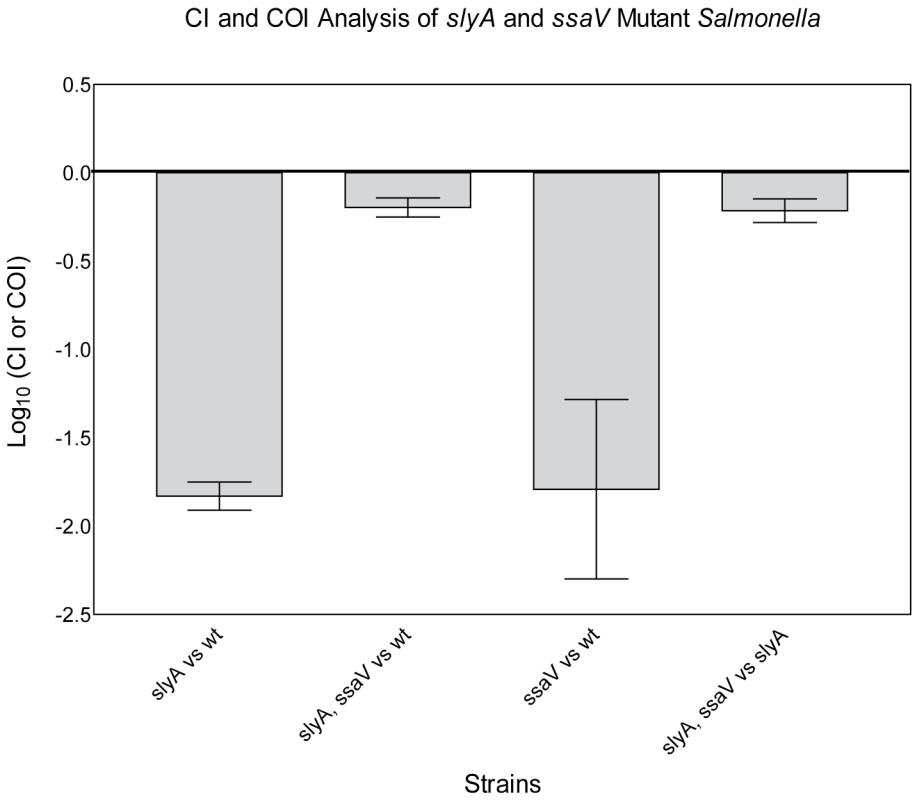

ssrB protected region
GCTGGCTGATATTGAAAATGCCGTATCGGCTGGAGAAAAAATCGATCAGTTAATTCACA
CATTAAAAGGCTGTTTAGGTCAAATAGGGCAGACTGAATTGGTATGCTATGTCATAGAC
ATTGAGAATCGCGTAAAAATGGGGAAAATCATCGCGCTGGAGGAACTAACCGACTTACG
CCAGAAAATACGTATGATCTTCAAAAACTACACCATTACTTAATATTATCTTAATTTTC
GCGAGGGCAGCAAAATGAAAGAATATAAGATCTTATTAGTAGACGATCATGAAATCATC
ATTAACGGCATTATGAATGCCTTATTACCCTGGCCTCATTTTAAAATTGTAGAGCATGT
TAAAAATGGTCTTGAGGTTTATAATGCCTGTTGTGCATACGAGCCTGACATACTTATCC
TTGATC
OmpR
SlyA
SsrB

Figure 7. Competitive infection assays. A double mutant carrying mutations in *ssaV* and *slyA* was analyzed by mixed infection with either *slyA* or *ssaV* mutant strains. The competitive infection indexes obtained for comparing an *ssaV*/*slyA* double mutant to either a *slyA* or *ssaV* mutant are statistically significant from the competitive indexes of the single mutant strain relevant in each case (95% confidence intervals).

Figure 7.


CI and COI Analysis of slyA and ssaV Mutant Salmonella
0.5
0.0
-0.5
-1.0
-1.5
-2.0
-2.5
Log10 (CI or COI)
slyA vs wt
slyA, ssaV vs wt
ssaV vs wt
slyA, ssaV vs slyA
Strains

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Conclusion and Summary

The research presented here in this dissertation has focused on understanding the molecular mechanisms of *Salmonella* pathogenesis. First, the ferritin-like Dps protein of *S*. Typhimurium was found to be required for oxidative stress resistance and virulence. Resistance to oxidative stress plays an important role in the ability of *Salmonella* to resist killing by host phagocytes and cause a productive infection. Pathogenic microorganisms can resist host-derived reactive oxygen species by avoidance, inhibiting production, production of scavengers, metabolic detoxification, or repair of damage. Iron sequestration is another important mechanism, since intracellular Fe(II) can catalyze the formation of highly toxic oxyradicals from hydrogen peroxide.

The ferritin-like protein Dps has been found in many eubacterial and archaebacterial species. Similar to ferritin, Dps can sequester iron atoms to prevent their participation in the formation of toxic reactive oxygen species (13). Additionally, some Dps homologues appear to condense chromatin into a microcrystalline array that may physically protect DNA from damage (12). In the pathogenic gram-negative bacterium *Salmonella*, *dps* expression is induced following internalization of the bacteria by macrophages (3, 10). The results of our studies unequivocally demonstrate a role of *S.* Typhimurium *dps* in oxidative stress resistance and virulence. The most likely mechanism is the sequestration of iron and prevention of iron-dependent oxidative DNA damage. Dps can be added to the list of evolutionarily conserved antioxidant proteins employed by *Salmonella* to resist killing by host phagocytes.

The second aspect of this work focused on the understanding of the physiological role of SlyA in *Salmonella* pathogenesis. Although oxygen is essential for the survival of

all aerobic organisms, mechanisms of defense against the toxic by-products of aerobic metabolism are equally important for survival. Bacteria are exposed to oxidative stress during periods of prolonged starvation, as a consequence of aerobic metabolism and also within professional phagocytes. This work demonstrates that SlyA is a global transcription factor that is essential for survival within an aerobic environment. Furthermore, these observations suggest a direct link between the PhoP/PhoQ two-component system and the transcriptional regulator, SlyA, however, the exact mechanism of this interaction is still unknown. We also demonstrate that the novel regulatory locus that is only found in *Salmonella* spp. and is absent in *E. coli*. This locus is required for *slyA* expression and a transposon insertion mutant in STM2359 shows increased sensitivity to hydrogen peroxide and attenuation for virulence in mice comparable to a *slyA* mutant. The complex regulatory network of gene expression presented here only demonstrates that the interplay and overlap between pathways enables *Salmonella* to mount an appropriate cellular response depending on the environmental conditions.

Previous studies have demonstrated that both SlyA and PhoP are required for survival within professional phagocytes (1, 6), however, the possible connection between SlyA and PhoP has not been elucidated. Microarray analysis comparing the genetic expression profiles of *slyA* and *phoP* mutants has enabled for the identification a possible mechanism of SlyA activation by PhoP/Q or loci regulated by it. Our data point to a possible mechanism by which PhoP/Q controls the activation state of SlyA and its ability to interact with target promoters. The further examination of selected loci by real time (quantitative) PCR, promoter fusions, and DNase I protection assays has demonstrated that both proteins are involved in their expression. Previous studies of Green *et* al. (7)

suggest that PhoP regulates *slyA* expression by directly inducing *slyA* transcription. The data presented here clearly shows that there is no significant difference in the number of *slyA* transcripts present in either a *phoP* or *phoP*-constitutive mutant as compared to wild type. These data suggest that PhoP does not induce SlyA transcription. Furthermore, the effect of PhoP on SlyA-dependent gene expression cannot be simply explained on the basis of *slyA* transcriptional activation by PhoP.

In the case of at least certain SlyA/PhoP-dependent genes examined thus far, SlyA appears to control transcription directly, while PhoP instead appears to modulate SlyA specificity. Our model of a possible SlyA-PhoP interaction is shown in Figure 8. We propose that an activated (phosphorylated) PhoP directly interacts with SlyA and causes a change in the SlyA protein that enables it to bind to target promoters, including those of previously known PhoP-dependence. Mutations in either *slyA* or *phoP* abolish *pagC* expression, which suggests that both proteins are required for *pagC* expression. The largest difference in *pagC* expression is seen when PhoP is phosphorylated and combined with a *slyA* mutation, which demonstrates that SlyA directly controls *pagC* transcription. The DNase I protection assays and the EMS assays also demonstrate that SlyA, but not PhoP, directly interacts with the *pagC* promoter. At least in the case of *pagC*, SlyA controls transcription while PhoP controls the SlyA specificity for the promoter. Future studies are needed to dissect the PhoP/SlyA interaction and also to confirm our model that SlyA and PhoP share overlapping regulons.

The fourth aspect of this work demonstrates the role of SlyA in the activation of SPI2 gene expression. The SsrA/SsrB two-component system of *S.* Typhimurium has been found to be required for *Salmonella* to cause systemic infection in mice and is

essential for survival in host macrophages (2, 5, 8, 9). Elegant research by Vazquez-Torres *et al.* suggests that SPI-2 may interfere with the trafficking of the NADPH oxidase to *Salmonella*-containing vacuoles thereby preventing phagocyte-dependent oxidative killing (11). Transcriptional regulation of the SPI2 locus has recently been found to be induced by another two-component regulatory system, EnvZ/OmpR (4). This finding illustrates that a coordinated bacterial response to an environmental stimuli involves a complex and intricate network of gene expression. Furthermore, this response can involve many regulatory systems that themselves can be activated by a variety of environmental stimuli.

The work presented here demonstrates that another important transcriptional regulatory protein, SlyA, is required for *ssrB* expression and subsequent expression of the SPI2 locus. We also demonstrate that activation of the SPI2 locus can occur in the absence of either SlyA or OmpR, however, *ssrB* expression is severely reduced when an *ompR* and *slyA* mutation are combined. The competitive infection data presented here also demonstrates that SlyA directly controls SPI2 activation. The results of these studies can only be explained if the products of these genes act dependently, and therefore SlyA regulates the expression of the SPI2 type-III secretion system genes *in vivo*. Our data also shows that activation of the SPI2 locus is greatest under conditions of low pH and low Mg^{2+}. This data suggests that the PhoP/PhoQ two-component regulatory system may be involved indirectly in *ssrB* expression, however further studies are needed to elucidate the exact mechanism of SPI2 activation by PhoP.

In conclusion, the work presented in this dissertation has added to what is known about the molecular mechanisms of *Salmonella* pathogenesis. The Dps protein has been

shown to be required for oxidative stress reistance and virulence and the data suggest that the protein functions by sequestering iron, thereby preventing its participation in Fenton-mediated oxidative DNA damage. The transcriptional regulatory protein, SlyA, has been shown to be required for survival in aerobic environments and its transcription is not significantly affected by other known oxidative stress loci. The observations that PhoP is involved in SlyA-dependent gene transcription suggest that these two systems may be intricately intertwined with one another. Future studies will help to elucidate the exact mechanism of the PhoP-SlyA interaction. Finally, this work demonstrates that SlyA plays a role in the expression of *Salmonella* pathogenicity island 2. The contribution of SlyA to the activation of this locus will be the focus of future experiments in this laboratory. Collectively, this dissertation has significantly contributed to the understanding of *Salmonella* pathogenesis.

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