SlyA, a Transcriptional Regulator of *Salmonella typhimurium*, Is Required for Resistance to Oxidative Stress and Is Expressed in the Intracellular Environment of Macrophages

NANCY BUCHMEIER,¹ STUART BOSSIE,² CHIN-YI CHEN,^{2†} FERRIC C. FANG,³
DONALD G. GUINEY,² AND STEPHEN J. LIBBY^{2*}

Department of Pathology¹ and Department of Medicine,² University of California, San Diego, La Jolla, California 92093-0640, and Division of Infectious Diseases, University of Colorado Health Sciences Center, Denver, Colorado 80262³

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Appropriate regulation of genes enables *Salmonella typhimurium* to adapt to the intracellular environment of the host. The *Salmonella slyA* gene is in a family of transcriptional regulators that may play an important role in this adaptation. We have previously shown that *slyA* mutant *Salmonella* strains are profoundly attenuated for virulence and do not survive in macrophages. In this study, we demonstrate that the expression of multiple *Salmonella* proteins is regulated by SlyA during stationary phase and during infection of macrophages. Both of these conditions also induced the expression of a *slyA::lacZ* transcriptional fusion. Expression of the *slyA::lacZ* transcriptional fusion increased 15-fold in stationary phase and was not dependent on the stationary-phase sigma factor, RpoS. *slyA* mutant *Salmonella* strains were sensitive to oxidative products of the respiratory burst, including hydrogen peroxide and the products of the redox cycling compound paraquat, but not to nitric oxide donors. These results suggest that the SlyA regulon is activated during infection of the host and is required for resistance to toxic oxidative products of the reticuloendothelial system.

slyA was originally identified as a gene required for *Salmonella typhimurium* virulence and for survival in macrophages (24) and was subsequently shown to belong to a family of low-molecular-weight transcriptional regulators (26). SlyA is required for the expression of a cryptic hemolysin in *Escherichia coli* K-12 strains, encoded by the *chyA* gene (29), but the presence of *chyA* in *Salmonella* has not yet been confirmed. SlyA has sequence homology with members of a family of transcriptional regulators which include EmrR, MarR, HprR, and PecS proteins, as well as a hypothetical protein (CY79) from *Mycobacterium tuberculosis* (26, 28). MarR and EmrR regulate a set of genes involved in multiple antibiotic resistance in *E. coli*, and homologous loci have been identified in *Salmonella*. The *marR* gene appears to be expressed in macrophages infected with *S. typhimurium* (33), but *marR* mutants are not attenuated for virulence in mice (32). PecS is a regulatory protein controlling the synthesis of pectinase and cellulase in *Erwinia chrysanthemi*, enzymes that enable *Erwinia* to infect fruit. The *Bacillus subtilis* HprR protein is required for resistance to hydrogen peroxide and regulation of sporulation. Thus, this family of regulators is required for adaptation of bacteria to varying environmental conditions.

Salmonella slyA mutants are profoundly attenuated for virulence in mice by intravenous, intraperitoneal, and intragastric routes of infection (10, 24). We have previously shown that an *slyA* mutant is unable to survive in the tissues of the reticuloendothelial system. The mechanism by which SlyA facilitates *Salmonella* survival and replication in macrophages is not well understood. In this study, we show that *slyA* mutants of *S. typhimurium* are hypersusceptible to reactive oxygen species

most likely encountered within the macrophage environment. We also demonstrate that *slyA* expression is induced during stationary phase and following *Salmonella* internalization by macrophages and that under these conditions, the expression of multiple *Salmonella* proteins is dependent on SlyA. These observations suggest that *slyA* may control the expression of genes required to resist oxygen-dependent antimicrobial systems of phagocytic cells.

MATERIALS AND METHODS

Bacterial strains, plasmids, and media. Bacterial strains used in this study are described in Table 1. *S. typhimurium* ATCC 14028s or derivatives were grown on Luria-Bertani (LB) or M9 glucose (0.2%) solid or liquid medium supplemented with penicillin (200 µg/ml), chloramphenicol (10 µg/ml), or tetracycline (10 µg/ml) when required.

Plasmids, genetic manipulations, and recombinant DNA methods. Construction of the *slyA* mutant has been reported previously (24). A *slyA::lacZ* reporter fusion was constructed by ligating a PCR fragment containing the first eight amino acids of the *slyA* coding region and 1 kb of upstream sequence into the *EcoRV* site of pBSSKII+ (Stratagene, La Jolla, Calif.). This fragment containing the *slyA* promoter was recombined into a promoterless *lacZ*-containing plasmid to create a *slyA::lacZ* transcriptional fusion. The *slyA::lacZ* fragment was subcloned into pSX34 (New England Biolabs, Beverly, Mass.), a derivative of pSC101, a low-copy-plasmid which is stable in *Salmonella* (9). The resulting plasmid, pSB1056, was introduced into *S. typhimurium* by electroporation. Expression of the *slyA::lacZ* fusion in the wild-type strain and in an *rpoS* mutant of *S. typhimurium* in logarithmic- or stationary-phase cells was quantitated by the method described by Miller (25).

To facilitate inducible *slyA* expression, a fragment containing *slyA* was placed under the transcriptional control of the arabinose operon contained on a plasmid derivative of pBAD18 (21). A fragment containing the *araC*-P_{BAD} promoter, and multiple cloning site of pBAD18, was excised with *Cla*I and *Fsp*I, treated with the Klenow fragment of DNA polymerase, and cloned into the *Sma*I site of pSX34. The resulting plasmid was designated pSL2570. A PCR-amplified 472-bp fragment containing the ribosome binding sequence, initiation codon, and translational stop codon of the *slyA* gene was cloned into the *Sma*I site of pSL2570, creating plasmid pSL2571. Plasmid DNA was introduced into *Salmonella* by electroporation.

³⁵S labeling of bacteria in culture and in macrophages. The pattern of proteins expressed in wild-type or *slyA* mutant bacteria was analyzed as previously described (5). Log-phase and stationary-phase *Salmonella* cells were labeled for 1 h with 50 µCi of [³⁵S]methionine (New England Nuclear, Boston, Mass.) per

* Corresponding author. Present address: Department of Microbiology, North Carolina State University, Box 7615, Raleigh, NC 27695-7615. Phone: (919) 513-1690. E-mail: slibby@mbio.ncsu.edu.

† Present address: Fox Chase Cancer Center, Philadelphia, PA 19111.

TABLE 1. Strains and plasmids used

Strain	Relevant features	Source or reference(s)
<i>S. typhimurium</i>		
ATCC 14028s	Wild type	Lab stock
SL2236	<i>slyA::pRR10ΔtraA</i> Pen ^r	24
SL2237	pSL2237 <i>slyA</i> in pSC101	24
SL2488	14028s pSB1056	This study
SL2463	<i>rpoS::tet</i> pSB1056	This study, 7
SL2570	SL2236 pSL2570	This study
SL2571	SL2236 pSL2571	This study
<i>E. coli</i> DH5α	<i>endA1 hsdR17(r_K⁻ m_K⁺) supE44 thi-1 recA1 pyrA(Nal^r) relA1 Δ(lacIAA-argF)U169 deoR[φ80dlacΔ(lacZ)M15]</i>	Lab stock
Plasmids		
pSX34	pSC101 derivative Cm ^r	New England Biolabs
pBAD18	<i>araC</i> -P _{BAD} plasmid	21
pSL2237	<i>slyA</i> in pSC101	24
pSL2570	pSX34: <i>araC</i> -P _{BAD} multiple cloning site	This study
pSL2571	pSX34: <i>araC</i> -P _{BAD} - <i>slyA</i>	This study
pSB1056	<i>slyA::lacZ</i> in pSX34	This study

ml. Bacteria were washed twice and solubilized by boiling in polyacrylamide gel electrophoresis (PAGE) sample buffer containing sodium dodecyl sulfate (SDS) and β-mercaptoethanol. *Salmonella* proteins synthesized during infection of J774 macrophages were prepared by infecting cells with log-phase bacteria for 15 min, washing the cells, and then adding gentamicin (12 μg/ml) to inhibit extracellular bacteria and cycloheximide (50 μg/ml) to prevent macrophage protein synthesis. The infected cells were initially incubated for 2 h, 50 μCi of [³⁵S]methionine was added, and the cells were incubated for an additional 2 h. The macrophage monolayers were then washed three times and lysed with 0.5% deoxycholate in the presence of protease inhibitors leupeptin, pepstatin, EDTA, and phenylmethylsulfonyl fluoride, plus DNase and RNase (5). Bacteria released from the macrophages were pelleted and solubilized by boiling in PAGE sample buffer. Proteins were separated on an SDS–12% polyacrylamide gel. Protein gels were fixed, dried, and exposed to X-ray film.

Measurement of *slyA::lacZ* activity in macrophages. Expression of a transcriptional *slyA::lacZ* fusion was measured in J774 macrophages as previously described (8, 18). Briefly, J774 macrophages were infected with *S. typhimurium* containing pSB1056 at multiplicity of infection of 5:1. Opsonized bacteria were centrifuged onto the macrophages to facilitate phagocytosis, incubated for 20 min at 37°C, and then washed three times in phosphate-buffered saline. Fresh medium containing 20 μg of gentamicin per ml was added to the cells. To measure *slyA::lacZ* expression, cells were lysed in 250 μl of 0.5% deoxycholate, and aliquots were removed to determine the number of viable bacteria by serial dilution and plating. β-Galactosidase activity was measured by using the chemiluminescent substrate AMPGD (23) from Tropix, Bedford, Mass. A detailed description of this method can be found in reference 8.

Measurement of susceptibility to reactive oxygen and nitrogen intermediates. (i) **Hydrogen peroxide.** Overnight cultures of the wild type, strain SL2236, and strain SL2237 (*slyA* mutant with pSC101:*slyA*) were diluted to 10⁷ CFU in phosphate-buffered saline and incubated with 400 μM hydrogen peroxide (Sigma, St. Louis, Mo.) at 37°C. At 30-min time intervals, aliquots were removed and the number of viable cells was determined by serial dilution and plating on LB agar.

(ii) **Paraquat.** Susceptibility to paraquat (methyl viologen; Sigma) was determined by a disk diffusion assay (4). Overnight cultures of strains 14028s, SL2570, and SL2571 were diluted in saline, and 10⁶ cells were spread on M9 glucose and allowed to dry. Ten microliters of 1 mM paraquat made in sterile water was spotted on a filter paper disk placed in the center of the plate, which was incubated overnight at 37°C. The zone of growth inhibition was measured in two axes relative to the disk and averaged. To complement the *slyA* mutation, overnight cultures containing plasmid SL2571 (*araC*-P_{BAD}-*slyA*) were incubated with 0.4% arabinose for 2 h, diluted, and plated onto M9–0.02% glucose plates with a 7-ml M9 agar overlay containing 0.4% arabinose and 10 μg of chloramphenicol per ml.

(iii) **Nitric oxide donors.** Susceptibility to *S*-nitrosoglutathione (GSNO) and 3-morpholinodisulfonimine (SIN-1; Sigma) was determined by a disk diffusion assay (4). GSNO was prepared by adding NaNO₂ in 1 M HCl to reduced glutathione (Sigma) and neutralizing with NaOH to pH 7.5. Fifteen microliters

FIG. 1. *SlyA* regulates protein expression in stationary phase and when *Salmonella* cells are in the intracellular environment of macrophages. *S. typhimurium* wild-type or *slyA* mutant cells were pulse-labeled with [³⁵S]methionine during logarithmic phase, stationary phase, or infection of J774 macrophages and proteins were electrophoresed on a 12% gel. Lane 1, wild type, log phase; lane 2, *slyA* mutant, log phase; lane 3, wild type, stationary phase; lane 4, *slyA* mutant, stationary phase; lane 5, wild type-infected J774 macrophages; lane 6, *slyA* mutant-infected J774 macrophages. Arrowheads denote changes in protein expression in wild-type versus *slyA* mutant cells in stationary phase or in macrophages.

of 500 mM nitric oxide donor compound was added to a 1/4-in. paper disk placed over a lawn of 10⁶ bacteria spread over M9 minimal agar with 0.2% glucose as described previously (12). The resulting zone of inhibition in six axes was measured after overnight incubation at 37°C.

RESULTS

***SlyA* regulates *Salmonella* protein expression during stationary phase and within macrophages.** The ability of *Salmonella* to survive in the host environment requires the coordinate regulation of a large number of genes (1, 2, 5, 6, 20). Regulation of genes expressed within host macrophages is controlled by several regulators, including PhoP/PhoQ (16, 17) and σ^S (RpoS) (8, 14), and inactivation of these loci results in reduced bacterial virulence. 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 parallel conditions encountered within host cells (8). The pattern of *Salmonella* proteins expressed by wild-type and *slyA* mutant *S. typhimurium* was determined during logarithmic phase, during stationary phase, or within J774 macrophages. Bacteria were labeled with [³⁵S]methionine under these three conditions, proteins were separated by SDS-PAGE, and labeled proteins were visualized by autoradiography. As shown in Fig. 1, the patterns of labeled proteins synthesized by wild-type and *slyA* mutant *Salmonella* were similar during the logarithmic phase of growth (lanes 1 and 2) but differed significantly during stationary phase (lanes 3 and 4). There were both enhanced and diminished levels of expression of proteins by the *slyA* mutant *Salmonella* compared to the wild

FIG. 2. A *slyA::lacZ* transcriptional fusion is induced in stationary phase and is not dependent on a sigma factor (RpoS). (A) Expression of the *slyA::lacZ* transcription fusion (pSB1056) was determined in log phase and in stationary phase by measuring the level of β -galactosidase by the method of Miller (25). (B) pSB1056 was transformed into an isogenic *rpoS* mutant, and β -galactosidase levels were measured.

type during stationary phase, indicating that SlyA may act as both a positive and a negative regulator.

To determine whether SlyA regulates *Salmonella* protein expression during infection of macrophages, logarithmically growing bacteria were used to infect macrophages, since no major differences between wild-type and *slyA* mutant proteins had been observed with log-phase bacteria. There were multiple differences between *slyA* mutant *Salmonella* and wild-type *Salmonella* in the pattern of proteins produced during infection of macrophages (Fig. 1, lanes 5 and 6). At least two proteins expressed in macrophages appear to be *slyA* dependent, and two macrophage-induced proteins appear to be negatively regulated by *slyA*. Collectively, these data indicate that SlyA exerts both positive and negative regulatory effects on gene expression during stationary phase and within macrophages.

The *slyA::lacZ* transcriptional fusion is expressed during stationary phase and within macrophages. (i) **Growth phase expression.** To determine whether *slyA* expression varies in different phases of growth, a *slyA::lacZ* (β -galactosidase) transcriptional fusion was constructed on a low-copy-number pSC101 derivative (pSB1056). This plasmid was transformed into wild-type *S. typhimurium* 14028s, and *lacZ* expression (in Miller units) was measured during logarithmic and stationary phases of growth. *slyA::lacZ* expression was very low during logarithmic phase (15 Miller units) but increased 15-fold in stationary phase (229 Miller units) (Fig. 2). To ascertain whether *slyA* expression is regulated by the stationary-phase sigma factor σ^S (RpoS), an isogenic *rpoS* *S. typhimurium* mutant was transformed with the *slyA::lacZ* plasmid, and the level of β -galactosidase expression was determined. The levels of *slyA::lacZ* expression were essentially identical in the two strains, demonstrating that *rpoS* is not required for *slyA* expression (Fig. 2).

(ii) **Macrophage expression.** *Salmonella* strains have been shown to express a specific subset of genes in response to the intracellular environment of the macrophage (1, 2, 5). To determine whether internalization by macrophages causes the induction of *slyA*, the expression of a *slyA::lacZ* fusion by wild-

FIG. 3. *slyA::lacZ* is expressed when *Salmonella* are in the intracellular environment of J774 macrophages. J774 macrophages were infected with logarithmically growing wild-type *S. typhimurium* harboring the *slyA::lacZ* fusion plasmid. At the times indicated, the level of β -galactosidase activity, determined by using a chemiluminescent substrate, was measured in triplicate wells of infected macrophages. Data are displayed as mean relative light units per bacterium plus standard deviation.

type *S. typhimurium* was measured following infection of J774 macrophages, using a chemiluminescent β -galactosidase substrate. Logarithmically growing cells, in which *slyA::lacZ* expression is present at low levels, were used to infect J774 macrophages, and the expression of *slyA::lacZ* was measured over the subsequent 6 h. *slyA::lacZ* expression increased 12-fold over the course of the macrophage infection (Fig. 3). The induction of *slyA* transcription was also visually detected by using a SlyA-green fluorescent protein transcriptional fusion (data not shown). These results show that the intracellular environment of the macrophage induces *slyA* expression.

***slyA* mutant *S. typhimurium* has increased susceptibility to reactive oxygen intermediates but not nitric oxide donors.** The *slyA* mutant *Salmonella* strain is profoundly attenuated for virulence by intraperitoneal, intravenous, and oral routes of infection in mice (10, 24). *slyA* mutants also are unable to survive and replicate in murine peritoneal macrophages from BALB/c mice. Nevertheless, *slyA* mutants are able to grow on minimal salts-glucose medium and on plaque bacteriophage P22 (which requires a complete lipopolysaccharide), and they survive normally during stationary phase. To identify specific stress conditions which may correspond to the host environment in which the function of SlyA is critical, we measured survival of the *slyA* mutant after exposure to reactive oxygen intermediates produced by the respiratory burst and reactive nitrogen intermediates produced by inducible nitric oxide synthase.

(i) **Hydrogen peroxide.** Stationary-phase cultures of wild-type and *slyA* mutant *S. typhimurium* (with or without the *slyA* gene on pSC101) were diluted, 10^7 CFU was exposed to 400 μ M hydrogen peroxide, and the number of viable cells was determined as a function of time. As shown in Fig. 4, the *slyA* mutant strain was killed by 400 μ M hydrogen peroxide, but the isogenic wild-type parent was not. The number of viable *slyA* mutant *Salmonella* declined by 3 orders of magnitude after 4 h of exposure, while the number of wild-type *Salmonella* re-

FIG. 4. *slyA* mutant *S. typhimurium* is sensitive to hydrogen peroxide. Hydrogen peroxide sensitivities of wild-type *S. typhimurium* 14028s, *slyA* mutant SL2236, and *slyA* mutant SL2236 with complementing plasmid pSL2237-pSC101::slyA were determined by incubating 10^7 bacteria with 400 μ M hydrogen peroxide for the times indicated and determining viable cells by serial dilution and plating on LB agar. The data represent one of three separate experiments.

remained essentially constant. Supplying the *slyA* gene in *trans* (SL2237) restored resistance to hydrogen peroxide killing.

(ii) **Paraquat.** The redox-cycling agent paraquat generates increased levels of intracellular superoxide radical ($O_2^{\cdot-}$). We determined the relative susceptibility of wild-type and *slyA* mutant *S. typhimurium* to paraquat in a disk diffusion assay. A disk containing 10 μ l of 1 mM paraquat was placed in the center of a lawn of 10^6 wild-type *S. typhimurium*, *slyA* mutant *S. typhimurium*, *slyA* mutant *S. typhimurium* carrying pSL2570, or *slyA* mutant *S. typhimurium* carrying cloned *slyA* under the control of the inducible *araC*-P_{BAD} promoter (pSL2571). The plates contained arabinose to induce *araC*-P_{BAD}-*slyA*. After overnight incubation at 37°C, the susceptibility to paraquat was quantitated by measuring the zone of growth inhibition. The *slyA* mutant *Salmonella* strain (SL2236) was markedly inhibited by 1 mM paraquat, while growth of the wild type was only slightly reduced (Fig. 5). Resistance of the *slyA* mutant to paraquat could be restored with an inducible clone of *slyA* (SL2566), while the presence of the pBAD18 vector alone had no effect (SL2565). Thus, *slyA* mutant *S. typhimurium* has enhanced susceptibility to both hydrogen peroxide and superoxide, and resistance can be restored by supplying the *slyA* gene in *trans*.

(iii) **Nitric oxide donors.** Susceptibility to nitric oxide was screened by using two compounds that possess inhibitory or bactericidal activity against *Salmonella* (12). GSNO is capable of both homolytic NO $^{\cdot}$ release and heterolytic NO $^+$ transfer (30). SIN-1 spontaneously generates peroxynitrite (OONO $^-$) under aerobic conditions. These reactive nitrogen species have been implicated in NO-related antimicrobial activity (12, 13). The *slyA* mutant *S. typhimurium* strain SL2236 does not differ significantly from the wild-type strain regarding susceptibility to these nitric oxide donors (Fig. 6), while two known nitric oxide-susceptible *S. typhimurium* mutants, with mutations of *metL* and *sodB* (11, 12), are susceptible. These results suggest that the attenuated virulence of *slyA* mutant *S. typhimurium* does not result from enhanced susceptibility to products of the host-inducible nitric oxide synthase.

FIG. 5. *slyA* mutant *S. typhimurium* is sensitive to paraquat. Wild-type *S. typhimurium* 14028s, *slyA* mutant SL2236, *slyA* mutant SL2565 with pSL2570-P_{BAD} vector, and a *slyA* mutant with pSL2571-*araC*-P_{BAD}-*slyA* were tested for sensitivity to 1 mM paraquat (2.5 μ g of total drug in the disk) by measuring the zone of growth inhibition after overnight incubation. The data represent one of three similar experiments.

DISCUSSION

This study was undertaken to examine the role of the *slyA* gene in *Salmonella* pathogenesis and resistance to the antimicrobial activity of macrophages. We have previously shown that *slyA* mutants are unable to survive and replicate in elicited murine peritoneal macrophages (24). We have also shown that *slyA* mutants are able to reach extraintestinal sites, namely, the liver, spleen, mesenteric lymph nodes, and Peyer's patches, a finding recently confirmed by others (10). Although *slyA* mutants are able to invade deep tissues, they are unable to proliferate efficiently within the reticuloendothelial cells of murine

FIG. 6. *slyA* mutant *S. typhimurium* is not susceptible to nitric oxide donors. Susceptibilities of wild-type and *slyA* mutant *S. typhimurium* and two known nitric oxide-sensitive (*metL* and *sodB*) *S. typhimurium* mutants to nitric oxide donors were determined by the disk diffusion assay. Disks containing GSNO or SIN-1 were placed on lawns of 10^6 bacteria spread over M9 minimal agar with 0.2% glucose and incubated overnight at 37°C. Data represent the mean zone of inhibition from measurements on six axes plus the standard error. The data represent one of three similar experiments.

hosts, suggesting that *Salmonella* strains are unable to withstand host cellular antimicrobial mechanisms in the absence of SlyA-regulated genes.

The present data indicate that SlyA regulates the expression of multiple *Salmonella* proteins in both a positive and a negative manner. SlyA-dependent protein expression was detected both during stationary-phase growth and during infection of macrophages but was not dependent on *rpoS*. Recent studies with *Salmonella rpoS* expression suggest that conditions encountered within host cells may partially resemble stationary phase (20); however, the patterns of SlyA-regulated proteins were significantly different in stationary phase and during infection of macrophages.

The *slyA* mutant *S. typhimurium* was very sensitive to both paraquat and hydrogen peroxide. Thus, SlyA joins the group of *Salmonella* genes which are required for resistance to oxidative stress: catalase genes *katE* and *katG*, *sod*, *rpoS*, and *oxyR* (19, 27). Hydrogen peroxide is a key component of the oxidative burst of host macrophages. Although it has been shown to be weakly microbicidal (3), production of hydrogen peroxide is an important component of the respiratory burst. In the bacterial cell, hydrogen peroxide can react with iron or copper ions to generate the toxic hydroxyl radical (OH) via the Fenton reaction (27, 31). The dioxygen-dependent toxicity of paraquat toxicity also depends on its accumulation in cells. Once intracellular, paraquat generates intracellular hydroxyl radical via generation of superoxide through repeated cycles of univalent reduction and autooxidation generating O_2^- and hydroxyl radical (15, 22). That the *slyA* mutant *S. typhimurium* is susceptible to both compounds suggests that some key cellular components regulated by SlyA are required to survive the accumulation of intracellular hydrogen peroxide, hydroxyl radical, and superoxide. *slyA* mutant *S. typhimurium* is not susceptible to nitric oxide donors, further demonstrating that it is reactive oxygen intermediates that are responsible for this phenotype.

Sequence analysis indicates that SlyA belongs to a family of transcriptional regulators (26). This family of proteins includes MarR and EmrR, which regulate the expression of membrane efflux systems that confer resistance to a wide range of toxic compounds (26, 28) and also exert some regulatory influence over genes involved in resistance to oxidative stress. Our data suggest that SlyA regulates critical genetic determinants which are required for adaptation to environmental stimuli encountered in the infected host. The intracellular expression of SlyA-regulated genes may constitute an inducible bacterial defense against reactive species generated by the NADPH-dependent phagocyte oxidase. This multicomponent cytochrome system converts molecular oxygen to superoxide radical, which in turn can spontaneously or enzymatically undergo dismutation to hydrogen peroxide. Both clinical and experimental observations suggest that the respiratory burst of phagocytic cells is an important component of the host defense against *Salmonella* infections. The further characterization of the components of the SlyA regulon promises to provide important insights into oxygen-dependent antimicrobial mechanisms in host-pathogen interactions.

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