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The transcription factor SlyA from *Salmonella* Typhimurium regulates genes in response to hydrogen peroxide and sodium hypochlorite

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Abstract

Salmonella Typhimurium is an intracellular pathogen that is capable of generating systemic fever in a murine model. Over the course of the infection, *Salmonella* faces different kinds of stressors, including harmful reactive oxygen species (ROS). Various defence mechanisms enable *Salmonella* to successfully complete the infective process in the presence of such stressors. The transcriptional factor SlyA is involved in the oxidative stress response and invasion of murine macrophages. We evaluated the role of SlyA in response to H_2O_2 and NaOCl and found an increase of *slyA* expression upon exposure to these toxics. However, the SlyA target genes and the molecular mechanisms by which they influence the infective process are unknown. We hypothesised that SlyA regulates the expression of genes required for ROS resistance, metabolism, or virulence under oxidative stress conditions. Transcriptional profiling in wild type and Δ*slyA* strains confirmed that SlyA regulates the expression of several genes involved in virulence [*sopD* (STM14_3550), *sopE2* (STM14_2244), *hilA* (STM14_3475)] and central metabolism [*kgtP* (STM14_3252), *fruK* (STM14_2722), *glpA* (STM14_2819)] in response to H_2O_2 and NaOCl. These findings were corroborated by functional assay and transcriptional fusion assays using GFP. DNA-protein interaction assays showed that SlyA regulates these genes through direct interaction with their promoter regions.

1. Introduction

Salmonella enterica serovar Typhimurium (*S.* Typhimurium) is a gram-negative, facultative anaerobe and generalist pathogen that is capable of causing bacteraemia, gastroenteritis, and systemic infection [1]. During its infective cycle, *Salmonella* is recognised by macrophages, neutrophils, and dendritic cells, which internalise and contain the bacterium in a *Salmonella* containing vacuole (SCV), where *Salmonella* faces several stresses, including low pH, low iron levels, and reactive oxygen and nitrogen species (ROS/RNS) [2].

The first ROS produced by phagocytic cells in a reaction catalysed by the enzyme NADPH oxidase is superoxide (O_2^-). Under acidic conditions, two molecules of O_2^- spontaneously react to form hydrogen peroxide (H_2O_2). In the presence of iron, H_2O_2 can generate the highly reactive hydroxyl radical (OH·) through the Fenton reaction [3]. Another enzyme involved in ROS generation is myeloperoxidase (MPO), which is mainly found in neutrophils and catalyses hypochlorous acid (HOCl) production from chloride (Cl^-) and H_2O_2 [4]. All of these ROS (O_2^-, HOCl, H_2O_2, and OH·) are highly reactive and oxidise important biological components, including lipids, proteins, and nucleic acids. Additionally, HOCl also chlorinates macromolecules [5].

Salmonella is able to detect and respond to environmental changes occurring during the infective process by modulating gene expression, allowing it to detoxify and adapt to stress conditions. A transcription factor involved in this process is SlyA, a member of the MarR transcription factor family, which recognises inverted repeats in DNA sequences; $TTAN_6TAA$ has been determined to be its consensus sequence [6–8]. SlyA regulates gene expression in response to a variety of molecules, including antibiotics, organic compounds, disinfectants, and the ROS-generating compounds paraquat and H_2O_2. In *Escherichia coli*

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and *S.* Typhimurium, SlyA acts as a virulence factor that is essential for replication and survival inside macrophages [9–11].

Most genes regulated by SlyA are thought to encode outer membrane and periplasm proteins, secreted proteins, and other proteins that are implicated in virulence and microbicide peptide resistance, including *nmpC*, *pagC*, *ugtL*, and *mgtB* [6, 7, 12-15], suggesting that the main role of SlyA in the protection against toxic compounds may lie in an alteration of the cell surface [9]. However, little is known about which genes are directly regulated by SlyA or their roles in bacterial virulence.

Despite the fundamental role of SlyA in resistance to toxic compounds presented by the host, especially those from the oxidative burst, little is known about the genes and metabolic pathways that may be modulated by oxidative stress induced by H_2O_2 and NaOCl. Thus, we hypothesised that SlyA regulates the expression of genes required for ROS tolerance, metabolism, and/or virulence under oxidative stress conditions. Transcriptomic analyses presented in this study suggest that SlyA from *S.* Typhimurium under oxidative stress both positively and negatively regulate the expression of various genes involved in processes that are important for bacterial survival and replication.

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2. Materials and methods

2.1 Bacterial strains and growth conditions

Bacterial strains used in this work are listed in Table 1. Cells were grown aerobically with agitation in Lennox broth (LB), with solid medium consisting of agar (20 g/L), and plates were incubated at 37°C. Dilutions (1:100) of overnight cultures were used to initiate growth. Where necessary, the growth medium was supplemented with the appropriate antibiotics. For ROS induction, bacterial strains were treated at OD_{600} ~0.4 with 1 mM H_2O_2 or 3 mM NaOCl for 20 min at 37ºC. The control cells received no treatment.

2.2 ROS treatment, RNA isolation, and RNA-seq analysis

Overnight cultures were diluted (1:100) and cells were grown to OD_{600} ~0.4 and received ROS treatment. After incubation with ROS, RNA was extracted using the acid–phenol method [16] and suspended in 30 µl of nuclease-free water. Intra-macrophage bacterial RNA was also extracted using this method. Additionally, 10^7 bacteria/ml grown in microaerophilic conditions were incubated with murine macrophages (Raw 264.7). After bacterial infection (see below), cells were incubated at 1 and 3 h post-infection and washed twice with PBS. RNA integrity was estimated from 1% agarose gels, and its concentration was determined spectrophotometrically. The cDNA library preparation and sequencing were performed at Macrogen Inc. (Seoul, Korea), using a TruSeq mRNA Library Prep Kit (Illumina, Inc.) for library construction, which was sequenced on an Illumina HiSeq 2500 system. Two independent biological replicates were performed. Raw data were filtered and trimmed using the PrinSeq lite 0.20.4 software, to ensure the quality of downstream analyses, as follows: Quality: -min_qual_mean 20 to filter reads of a quality below 20; Trimming: -trim_qual_left 20 -trim_qual_right 20 to trim bases of a quality below 20 at

both extremes; Ambiguity: -ns_max_n 0 to filter reads with Ns; Low complexity: DUST method -lc_method dust -lc_threshold 7 to filter reads with a complexity score above 7 [17]. An average of 10.5 million reads were analysed per sample with an average coverage of 325X. Gene expression levels were estimated using the EDGE-pro v1.3.1 software (Estimated Degree of Gene Expression in PROkaryotes; Johns Hopkins University CCB, Maryland, USA) [18] by mapping reads against the *Salmonella enterica* subsp. *enterica* serovar Typhimurium strain 14028s reference genome (GenBank: GCA_001558355.1). The EDGE-pro software employed uses Bowtie2 [19] to map reads to gene models; it accommodates reads that map to overlapping reading frames and other features exclusive to prokaryotes. The resulting matrix of counts was used to estimate differential gene expression using a negative binomial normalisation method as implemented in the DESeq2 Bioconductor Package [20]. Fold-changes are expressed as $\log_2$ changes with an FDR of < 0.1. RNA-seq data generated as part of this study were deposited in the NCBI SRA database under accession numbers SRR5192881 and SRR5192882 (Bioproject PRJNA357075).

2.3 Quantitative RT-PCR

Total mRNA was treated with DNase I, and cDNA was generated using the M-MLV Reverse Transcriptase (Promega), following the manufacturer's instructions. The expression of seven genes was measured: *sopD*, *sopE2*, *hilA*, *fruK*, *glpA*, *kagtP*, and *pagC*. Primers used for qRT-PCR are listed in Table 2. Relative quantification was performed using the Brilliant II SYBR Green QPCR Master Reagent Kit and the M×3000P detection system (Stratagene). The reaction mixes were prepared according to the manufacturer's instructions, with reaction conditions as follows: 10 min at 95°C followed by 40 cycles of

30 s at 95°C, 30 s at 55°C and 30 s at 72°C, followed by a melting cycle from 65°C to 95°C to check for amplification specificity. Amplification efficiency was calculated from a standard curve constructed by amplifying serial dilutions of RT-PCR products for each gene. These values were used to obtain the fold-change in expression for the gene of interest normalised using *talB* levels, as described previously [21].

2.4 Construction of GFP-transcriptional fusions

The promoter regions of selected genes (*fruk*, *sopD*, *glpA*, *kgtP*, *sopE2*, and *hilA*) were cloned into the pGLO plasmid (Bio-Rad). Briefly, promoter regions (Table 2) were PCR-amplified and digested in parallel with plasmid pGLO using the restriction enzymes *Bmt*I and *Age*I (underlined sequences in Table 2, incorporated in primers). After ligation, the plasmids were used to transform electrocompetent *E. coli* Top10 cells. Correctly ligated plasmid was identified using PCR, sequenced, and transformed into wild type (WT) and Δ*slyA* strains.

2.5 Reporter Activity

Reporter activity was measured in strains containing the promoter construct as well as in control strains containing an empty vector. Strains were grown to OD_{600} ~0.4, centrifuged at 4400 rpm for 10 min, and suspended in 5 ml 1× PBS. Cultures were then split into three aliquots, then treated with the toxic compounds (H_2O_2 or NaOCl). Fluorescence was measured in 300-µl samples every 3 min for 45 min using a TECAN Infinite 200 PRO Nanoquant microplate reader (excitation, 395 nm; emission, 509 nm). Emission values were normalised to optical density. Specific fluorescence intensities were calculated as follows [22]: $(\Delta\text{fluorescence}/\Delta\text{OD})_{\text{construct}} - (\Delta\text{fluorescence}/\Delta\text{OD})_{\text{empty vector}}$.

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157 *2.6 Protein purification*

158 His-tagged SlyA for electrophoretic mobility shift assays (EMSAs) was purified
159 using the Protein Spin Miniprep His-tagged kit (Zymo Research). *E. coli* BL21 cells
160 carrying plasmid pET-TOPO-*slyA* were grown in 10 ml of LB medium supplemented with
161 ampicillin (100 μg/ml) to OD_{600} ~0.4. Protein overexpression was induced by adding 1 mM
162 IPTG and further growth for 4 h, after which the manufacturer's protocol for the Protein
163 Spin Miniprep His-tagged kit was followed.

164

165 *2.7 DNA binding assay*

166 Non-radioactive EMSAs were performed using increasing amounts of purified SlyA
167 that was incubated with 50 ng of PCR product(s), and DNA was evaluated in binding
168 buffer (100 mM Tris-Cl [pH 7.5], 10 mM EDTA, 100 mM NaCl, 10 mM $MgCl_2$, 50%
169 glycerol, and 50 mM DTT) for 20 min at room temperature (RT). Reaction mixtures were
170 immediately loaded onto 6% native polyacrylamide gels, which were run at 90V for 1.75 h
171 at 4ºC. DNA-protein complexes were visualised using ethidium bromide staining.
172 Polynucleotides used in EMSAs were generated using PCR (see Table 2). PCR fragments
173 were generated using primers designed for the promoter region of the genes; all promoter
174 regions were 300 bp long. A 124-bp *ompX* gene promoter region was used as a negative
175 control for the interaction.

176

177 *2.8 Determination of total cellular thiols*

178 Total thiol content was quantified using Ellman's reagent [5,5′-dithiobis-(2-
179 nitrobenzoic acid); DTNB, Sigma-Aldrich] by following TNB^{-2} generation at OD_{412}, as

previously described [23] . Values were normalised to mg of protein. Cells were grown to OD_{600} ~0.4, then treated with the toxics. After exposure to the toxic compounds, 500 µl of the culture were withdrawn and pelleted by centrifugation for 5 min at 12,000 *g*. The supernatant was discarded and the pellet suspended in fresh buffer (0.1 mM DTNB, 5 mM EDTA, 50 mM pH 8 Tris-HCl, and 0.1% SDS) and incubated for 30 min with agitation at 37ºC. The samples were centrifuged, and the absorbance of the supernatant was measured at 412 nm and corrected by the absorbance of the sample without DTNB.

2.9 Determination Pyruvate Kinase Activity

The pyruvate kinase activity was measure in cell lysates of study strains using the Pyruvate Kinase Assay Kit (Cayman Chemical). Cells were grown to OD_{600} ~0.4 and then ROS were induced; control cells received no treatment. The culture was centrifuged at 4,400 rpm for 10 min and the pellet was resuspended in 1 ml of cold 1× PBS at pH 7.4. The cells were then lysed by sonication and centrifuged at 14,000 rpm at 4°C for 30 min to remove the cellular debris. The pyruvate kinase activity measure was performed following the manufacturer's instructions.

2.10 Determination NAD^+/NADH levels

The NAD^+/NADH Cell-Based Assay Kit (Cayman Chemical) was used to determine NAD^+/NADH levels. Cells were grown to OD_{600} ~0.4, and then received ROS treatment. The cultures were centrifuged and the pellet was lysed, and NAD^+/NADH measurement was performed following the manufacturer's instructions.

2.11 Congo red binding assay

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The Congo red binding assay followed the protocol of Weatherspoon-Griffin and Wing [24], with modifications. The different strains were grown in LB medium to an OD_{600} ~0.4 and split in three aliquots of 5 ml each. Cells were centrifuged for 5 min at 4,400 rpm, the supernatant was discarded, and the pellet was suspended in 5 ml LB 0.1% Congo red. The strains were treated with the toxics. After centrifugation for 5 min at 4,400 *g*, the pellet was suspended in 5 ml 25% ethanol and incubated for 2 min, before a second centrifugation step under the same conditions. Finally, the supernatant was quantified at 498 nm. The amount of cells binding Congo red was measured against a standard curve of LB 0.1% Congo red serial dilutions.

2.12 Determination of biofilm formation

Biofilm formation was determined as previously described [25], with some modifications. Briefly, 1 ml of culture medium containing each of the different strains was centrifuged at 14,000 rpm for 1 min, and the pellet was re-suspended in 1 ml LB. Next, 100 µl bacteria were inoculated into a 96-well plate (by octuplicate) and incubated at 37ºC for 4 h. The medium was removed and 200 µl methanol were added. Bacteria were fixed by incubating the samples in methanol overnight. Following this, 125 µl 0.1% crystal violet was added to each well, and the plate was left to incubate at room temperature for 15 min. Crystal violet was removed in three washing steps with H_2O. After adding 125 µl 30% acetic acid to each well, the plate was again incubated for 15 min at room temperature. Samples were transferred to a new 96-well plate, and their absorbance was measured at 550 nm. The percentage of biofilm formation was calculated relative to the control WT strain, which was arbitrarily set as 100%.

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2.13 Determination of protein carbonylation

Cells were grown to OD_{600} ~0.4 before adding treatment. Cultures were centrifuged and pellets were suspended in Tris-HCl buffer (20 mM, pH 7.0). After further centrifuging at 12,000 *g* for 3 min, 50 µl streptomycin sulphate (50 mg/ml) were added, and samples were incubated for 5 min at RT before centrifuging at 12,000 *g* for 3 min. The precipitate was discarded. Three volumes of 10 mM 2,4-dinitrophenylhydrazine prepared in 2 M HCl were added to the supernatant, and the mixture was incubated for 1 h at RT with repeated vortexing every 15 min. Proteins were precipitated by incubating with two volumes of 20% trichloroacetic acid (TCA) (w/v) for 30 min at RT. After centrifugation at 12,000 *g* for 5 min, the supernatant was carefully removed. Pellets were washed five times with a mixture of ethyl acetate: ethanol (1:1), until the yellow colour faded. Finally, pellets were suspended in 750 µl of a solution of 6 M guanidine hydrochloride with 10 mM DTT, and the absorbance at 370 nm was measured. Subsequently, carbonyl contents were determined using the molar extinction coefficient $\varepsilon = 22\ mM^{-1}\ cm^{-1}$. The protein concentration was determined concurrently using the Bradford method.

2.14 Determination of thiobarbituric acid reactive substances

To determine the thiobarbituric acid reactive substances (TBARS), strains were grown to OD_{600} ~0.4 before inducing ROS. Cells were centrifuged at 12,000 *g* for 3 min. Pellets were suspended in 0.5 ml PBS 1× with 0.1 mM DPBS and sonicated three times for 30 s. Samples were then centrifuged at 13,000 *g* for 10 min, and the supernatant was transferred to a 2.0-ml microcentrifuge tube. Next, 1 ml 20% (w/v) TCA was added to precipitate proteins, and the mixture was incubated for 1 h at RT. Subsequently, samples

were centrifuged at 13,000 *g* for 20 min. The supernatant was transferred to a conical 15-ml tube and mixed with 1.5 ml 0.1 M HCl solution containing TBA 1% (w/v) and 10 mM DPBS. The samples were heated to 100°C for 1 h. The heated mixture 1.5 ml was transferred to a conical tube containing 1.5 ml butanol. After cooling, the sample was vortexed, followed by centrifugation at 4,000 *g* for 10 min. The absorbance of the organic phase was then measured at 535 nm. TBARs were determined using the molar extinction coefficient ε = 156 $mM^{-1}cm^{-1}$. The protein concentration was determined concurrently following the Bradford method.

2.15 Determination of reactive oxygen species

Bacterial strains were grown to an OD_{600} ~0.4 before adding H_2O_2 or NaOCl. Intracellular ROS levels were measured using the oxidation sensitive H_2DCFDA probe, as previously described [26] with minor modifications. Cells were incubated with 10 μM H_2DCFDA. Fluorescence was measured from treatment onset using a TECAN Infinite 200 PRO Nanoquant microplate reader (excitation, 480 nm; emission, 520 nm). Emission values were normalised to the optical density of treated and untreated strains, and cells with dimethyl sulfoxide (DMSO) were used as a blank. Measurements were taken every 5 min for a total period of 100 min. To calculate intracellular ROS, all possible noise that could affect the fluorescence was subtracted from the respective strain using DMSO. The difference in fluorescence was calculated and divided by the elapsed time. Finally, the values thus obtained were normalised to the difference in growth (OD) over the respective time.

2.16 Gentamicin protection assays

Infection assays were performed using strains *S.* Typhimurium 14028s and its isogenic derivatives Δ*slyA* and Δ*slyA/pBR*::*slyA*. Briefly, 10^7 bacteria/ml grown in microaerophilic conditions were centrifuged (13.000 rpm, 5 min) and resuspended in 1 ml of cell culture medium DMEM supplemented with 10% FBS. Then, 100 µl of the suspension were added to each well of a 96-well-plate containing cell monolayers of Raw 264.7 macrophages. The multiplicity of infection was 100:1 (bacteria:cell). After incubation for 1 h in 5% CO_2 at 37°C, cells were washed twice with sterile PBS and incubated for 2 h with 100 µl of cell medium plus 250 µg ml^{-1} gentamicin to kill extracellular bacteria. After 2 h, the medium was removed and the cells were washed twice with PBS. At this point, the cells contained in three of the wells were lysed with sodium deoxycholate (0,5% w/v in PBS), 10-fold serial diluted in PBS, and plated onto LB agar plates to finally determine the colony-forming units (CFU) after incubating 16 h at 37°C.

3. Results

3.1 Role of SlyA in response to oxidative stress

To analyse the role of SlyA in the transcriptional response to oxidative stress produced by H_2O_2 and NaOCl, the *S.* Typhimurium SlyA regulon was identified using RNA-seq analysis. Expression profiles were measured in WT and Δ*slyA* exposed to 1 mM H_2O_2 or 3 mM NaOCl, and compared to the respective strains without treatment. The chosen concentration corresponds at the minimal inhibitory concentration (MIC) of Δ*slyA*. Under these concentrations, a physiological effect was observed in both strains (data not shown), such as a change in behaviour growth, sensibility, survival, and ROS accumulation. The results were validated by selecting seven genes and measuring their

298 expression using qRT-PCR (see Methods). The selected genes are involved in gene
299 expression required for ROS tolerance, metabolism, and/or virulence, which is important
300 for *Salmonella* survival and replication under conditions found in the infective process.

301 To understand which metabolic pathways are altered under oxidative stress
302 conditions, we bioinformatically assayed the genes obtained by RNA-seq using the KEGG
303 database (*Salmonella enterica* serovar Typhimurium 14028s KEGG). A bioinformatics
304 assay of the genes obtained by RNA-seq indicated that these genes are implicated in
305 various metabolic pathways involved in bacterial survival and replication, including amino
306 acid metabolism, glycolysis, ribosomal metabolic pathways, two component systems, and
307 the pentose phosphate pathway (Fig. 1). The percentage of genes represents the total
308 number of genes that are differentially expressed in the RNA-seq assay.

309 Several transcriptional changes occurred in WT under H_2O_2 oxidative stress,
310 including changes in the expression of 68 genes (Supplementary Fig. 1A), such as genes
311 involved in ribosomal and metabolic pathways that are activated in different environments,
312 and in the secondary metabolite biosynthesis. Under stress caused by NaOCl, WT showed
313 transcriptional changes in the expression of 62 genes (Supplementary Fig. 1B). The main
314 pathways altered were metabolic and included the metabolism of nucleotides, sugars and
315 amino-sugars, phosphotransferase systems, secondary metabolite biosynthesis, and
316 metabolism pathways that are regulated in different environments (data not shown).
317 Twenty-one genes showed a change in expression under both stress conditions in the WT
318 strain (Supplementary Table 1). Among these, the *slyA* gene (STM14_1742) was positively
319 regulated, demonstrating a four-fold increase in expression under both H_2O_2 and NaOCl
320 oxidative stress.

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Some of the genes for which expression was altered under both conditions in WT strains were also identified in global transcriptomic analyses performed on bacteria rescued from phagocytic cells, as well as by *E. coli* and *Salmonella in vitro* analyses of oxidative stress generated by chlorinated species and peroxide [27-30]. One of the genes that coincides with our analysis is *pagC*, which is a gene related to SPI-2, and it is positively regulated under NaOCl stress. Additionally, we found a gene that encodes a putative cysteine synthase, which is related to cysteine biosynthesis and is positively regulated under H_2O_2 oxidative stress. This is consistent with a report by Wang et al. [28], who found that most of the genes related to cysteine biosynthesis were positively regulated under H_2O_2 treatments in *E. coli*. However, genes involved in *de novo* synthesis, such as *aroH*, which is essential for aromatic amino acid synthesis, were negatively regulated under both stress conditions, which is consistent with Eriksson *et al.*'s findings [29] during the intracellular *Salmonella* growth. This downregulation could decrease both virulence and survival of the bacteria under such stress conditions. Other genes that were positively regulated under NaOCl stress play a role in the metabolism of glucoronate (STM14_3797), an important source of carbon for bacterial growth under stress conditions.

In contrast to previous findings [30], our transcriptomic analysis of the WT strain revealed that under NaOCl oxidative stress, the PTS *manXYZ* system is positively regulated, which is a beneficial change for the bacteria. This difference in expression patterns may be because of differences in the concentrations of toxic (230 ppm vs. 130 or 390 ppm), the exposition time of the treatment (20 min vs. 10 min or 30 min), or bacterial strains used (*Salmonella enterica* 14028s vs. LT2 or PT4). Another significant difference in the reported data is the negative regulation of the multidrug resistance operon *marRAB*, which is inactive under both stress conditions evaluated. This can be attributed to the

compensatory activity of other genes that contribute to resistance to these toxic compounds (H_2O_2 or NaOCl), as in the case of *slyA*. Finally, we observed positive regulation of the *cadB* gene, which is involved in cadaverine transport. The *cadB* gene is induced under NaOCl oxidative stress, suggesting an increase in the acidic conditions in the medium [31]. This is consistent with observations by Eriksson *et al*. [29], who found an increased expression of this gene under the conditions found inside the macrophage.

For the Δ*slyA* strain, 23 genes demonstrated altered expression levels under H_2O_2 oxidative stress compared to control conditions (Supplementary Fig. 1C). These were mainly involved in propanoate metabolism, secondary metabolism, antibiotics metabolism, and amino acid biosynthesis. Under NaOCl stress, there was no significant alteration in gene expression compared to control conditions in Δ*slyA*.

3.2 SlyA-dependent genes differentially expressed after H_2O_2 treatment

RNA-seq experiments did not find any significant differences in global gene expression levels between WT and Δ*slyA* under control conditions, suggesting that in the absence of oxidative stress, both strains behave similarly. Under control conditions, growth and survival were comparable. However, under H_2O_2 stress, expression levels differed for 94 genes. This included alterations in the same metabolic pathways mentioned above (metabolism of nucleotides, sugars, and amino-sugars, phosphotransferase systems, secondary metabolic biosynthesis, and metabolism pathways that are regulated in different environments), as well as several pathways involved in the infective process, specifically, the bacterial secretion system and invasion of epithelial cells (Fig. 1A and 2A). Presumptive genes involved in these pathways include *sopD*, *sopE2*, and *hilA*, which

368 belong to pathogenicity island-1 (SPI-1) and are negatively regulated by SlyA. Rather than
369 suppression, the glycolytic pathway was activated under H_2O_2 stress, and we determined
370 that SlyA positively regulates the *fruK* gene that encodes the enzyme 1-
371 phosphofructokinase, which converts fructose 1-phosphate into fructose 1,6-bisphosphate.
372 To validate genes showing differential expression in RNA-seq analysis, we chose these
373 four genes and performed a relative quantification of all genes under oxidative stress.
374 Significant results were obtained for all conditions compared to the WT. The first gene
375 involved in bacterial virulence that was analysed was *sopD*, which encodes a secreted
376 effector protein. Its expression levels were higher in the Δ*slyA* than in WT under H_2O_2
377 conditions, with levels ranging from a ~0.6-fold increase in the presence of the toxic, to a
378 ~2.7-fold increase under control conditions (Fig. 3A). In accordance with the RNA-seq
379 analysis, where expression levels in WT showed a $\log_2$ fold-change of −2.2 compared to
380 the mutant, suggesting that *sopD* is downregulated by SlyA.

381 The *sopE2* gene encodes a type III-secreted effector protein. Its expression levels in
382 Δ*slyA* are ~0.5 times higher than those of WT under stress condition, and ~2.2 times those
383 of WT under control conditions (Fig. 3B). In the RNA-seq analysis, *sopE2* transcription
384 levels showed a $\log_2$ fold-change of −2.5 in WT compared to Δ*slyA* under H_2O_2 oxidative
385 stress. This suggests that SlyA might down-regulate *sopE2* in all conditions.

386 The last gene involved in virulence is *hilA*, which encodes an invasion protein
387 regulator. The *hilA* expression levels were higher in Δ*slyA* than in WT under study
388 conditions: under control conditions, they were 1.6-times the level of WT expression, in the
389 presence of H_2O_2, and 1.4-times the level of WT expression (Fig. 3C). Consistent with
390 these results and under this last condition, RNA-seq analysis showed a −2.8 $\log_2$ fold-

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391 change in *hilA* expression levels in the WT compared to Δ*slyA*. These results suggest that
392 *hilA* is downregulated by SlyA.

393 Finally, relative *fruK* gene expression showed a significant ~0.5-fold decrease in
394 Δ*slyA* in evaluated conditions (Fig. 3D). In the RNA-seq analysis, *fruK* showed changes
395 after H_2O_2 treatment, with a $\log_2$ fold-change of 2.96 in WT compared to Δ*slyA*
396 (Supplementary Tables 2 and 3). These results suggest that *fruK* is upregulated by SlyA.

397 To demonstrate that these effects resulted from a deletion of *slyA*, we performed
398 assays with a strain that has the *slyA* gene episomal vector (Δ*slyA*/pBR::*slyA*). The results
399 showed that the behaviour in this strain is similar to the WT under all conditions.

400

401 *3.3 SlyA-dependent genes differentially expressed after NaOCl treatment*

402 Transcriptional differences between both strains under NaOCl stress were found for
403 111 genes, which are also involved in the biochemical pathways previously mentioned,
404 including those involved in the infective process and central metabolism, as well as
405 glycerophospholipid and quorum sensing pathways (Fig. 1B and 2B). Within these
406 pathways, we found that the genes *glpA* and *kgtP*, which are involved in the central
407 metabolism of bacteria, were positively and negatively regulated by SlyA, respectively.
408 Moreover, the *pagC* gene that encodes a membrane protein involved in bacterial virulence
409 is positively regulated by SlyA. We chose these three genes to validate the RNA-seq
410 analysis, and performed a relative quantification of all genes under oxidative stress induced
411 by NaOCl.

412 The *glpA* gene encodes the anaerobic glycerol-3-phosphate dehydrogenase subunit
413 A, which is involved in glycerophospholipid metabolism. *glpA* expression levels were

414 significantly decreased in the Δ*slyA* strain under all evaluated conditions, demonstrating
415 ~0.6-fold suppression compared to WT in the absence of stress, and a ~0.5-fold suppression
416 in the presence of NaOCl (Fig. 3E). This is consistent with RNA-seq analysis results,
417 where, in the presence of NaOCl, *glpA* expression in WT showed a $\log_2$ fold-change of 3.6
418 compared to the mutant, suggesting that *glpA* is upregulated by SlyA.

419 The last gene involved in central metabolism is *kgtP*, which encodes the alpha-
420 ketoglutarate transporter. Its expression was significantly higher in the mutant than the WT
421 under this condition (two-fold increase; Fig. 3F). The RNA-seq analysis showed a −2.7 $\log_2$
422 fold-change in WT compared to the Δ*slyA* in NaOCl treatment. These results suggest that
423 *kgtP* is downregulated by SlyA under evaluated conditions.

424 Finally, relative *pagC* gene expression showed a significant decrease of ~0.9-fold in
425 Δ*SlyA* in this condition, suggesting that SlyA positively regulates *pagC* expression (Fig.
426 3G). In the RNA-seq analysis, *pagC* showed changes after NaOCl treatment, with a $\log_2$
427 fold-change of 2.6 in WT compared to the mutant (Supplementary Table 3). These results
428 are consistent with the regulation shown by SlyA in previous research [27].

429 Overall for H_2O_2 and NaOCl, we observed significant differences compared to
430 control, and these differences may be because of the affinity with which SlyA is bound to
431 the potential promoter regions of the targets (see below). Additionally, qPCR measures the
432 relative expression of the specific genes so the sensitivity is greater for detecting changes.

433 To demonstrate that these effects resulted from a deletion of *slyA*, we performed
434 assays with a strain that has the *slyA* gene episomal vector (Δ*slyA*/pBR::*slyA*). The results
435 showed that the behaviour in this strain was similar to the WT in all conditions. However,

436 *kgtP* and *pagC* gene expression showed a slight increase in control and NaOCl, respectively
437 (Fig. 3F and 3G).
438
439 *3.4 Influence of SlyA in bacterial physiology*
440 Although there was no transcriptomic analysis of SlyA under the study conditions,
441 the SlyA regulon has been extensively studied and we observed certain similarities with
442 previous research, showing different SlyA-regulated genes involved in virulence, multidrug
443 resistance, outer membrane proteins, system of two components, and metabolism. Within
444 the virulence coincident genes, we determined that *pagC* is positively regulated by SlyA,
445 which is consistent with previous reports [7, 13, 14]. However, proteomic studies of
446 *Salmonella* under oxidative stress induced by H_2O_2 [32] revealed an important inhibition in
447 the expression of proteins belonging to SPI-1. This included OrgA (STM14_3469), which
448 is involved in promoting cell invasion, the SopE2 effector protein, which was negatively
449 regulated under H_2O_2 stress, and the HilA regulatory protein, which is consistent with the
450 results obtained. For genes involved in multidrug resistance, we found two genes, *ydhJ* and
451 *ydhI* (STM14_1740; STM14_1741), which are transcribed divergent to *slyA* and are
452 negatively regulated by SlyA. Additionally, the *marA* gene that codes for a transcriptional
453 regulator involved in multiple resistance to antibiotics was shown to be regulated
454 negatively, which is consistent with results obtained by Navarre [7]. The participation of
455 SlyA in the regulation of outer membrane permeability has been widely described, and it
456 positively regulates different porins such as OmpC, OmpF, OmpD, and NmpC [6, 7, 33].
457 These studies are in agreement with our results that gene *nmpC* (STM14_1898) is regulated
458 by SlyA. We also determined that the gene codes for OmpN porin, which is also down-
459 regulated, suggesting that SlyA contributes to the membrane permeability balance. SlyA

involvement in two-component system (TCS) regulation has been previously discussed, suggesting that SlyA regulates the locus of *ssrAB*, a TCS that is responsible for controlling genes present in SPI-2 [7, 34]. SlyA can also activate *phoP* and other genes controlled by the PhoPQ TCS, and we revealed different loci regulated by SlyA, including *pmrA and rstA*, which both belong to a TCS that is involved in resistance to antimicrobial peptides and stress, respectively [13]. Thus, our results suggest a different target for the NarX / NarL TCS including *narJ* (STM14_2130), *narH* (STM14_2131), *narG* (STM14_2132), and *narK* (STM14_2134), which are positively regulated by SlyA. We also found two genes that encode response regulators that are regulated by SlyA *yhjB* (STM14_4338) and *creB* (STM14_5509), which are involved in citrate fermentation and phosphate metabolism, respectively. Additional SlyA target genes that were identified are involved in amino sugar and nucleotide sugar metabolism such as *nanK* (STM14_4027), *nanE* (STM14_4028), *nanT* (STM14_4029), and *nanA* (STM14_4030), and operon members that are responsible for obtaining energy from sialic acid. This is of particular importance because the amino sugars, apart from possessing a structural component in the formation of peptidoglycan in bacteria, are an important source of carbon and nitrogen. Our results are consistent with a previous analysis by Spory [33] in which the proteomics analysis showed induction of NanA and NanE proteins by SlyA.

3.5 Direct binding to promoter regions of metabolism and virulence genes

To determine whether SlyA regulates *sopD*, *sopE2*, *hilA fruk*, *glpA*, *kgtP*, and *pagC* genes *in vivo*, the promoter activity of each gene was evaluated using the vector pGLO in WT and Δ*slyA* strains under conditions of oxidative stress. The pBAD promoter was cleaved, and the *gfp* reporter activity was placed under the control of the promoter of the

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484 respective gene to be evaluated (Fig. 4I). Additionally, we aimed to demonstrate direct
485 binding of SlyA to each promoter. To achieve this, we first performed an *in silico* search
486 using the consensus sequence 5'-TTWGBAWTBWAA-3' (B = C, G or T; W = A or T) to
487 identify potential binding sites for the protein SlyA in each promoter region. The potential
488 binding sites of SlyA are listed in the Table 3 [6]. The direct interaction of SlyA with the
489 promoters was assessed by testing DNA-protein interaction in an EMSA. For the analysis
490 of each gene, a polynucleotide fragment of approximately 300 bp, which included the SlyA
491 binding sites of the relevant promoter regions, was incubated with increasing
492 concentrations of purified SlyA protein. A 120 bp-fragment of the *ompX* promoter region
493 was used as a negative control (Fig. 4II).

494 Under the *sopD* and *sopE2* promoter, reporter activity showed slight significant
495 differences between Δ*slyA* and WT in the presence of H_2O_2, while the activity was less in
496 the mutant than in WT. This is consistent with the qPCR analysis where the variation of
497 this transcript in this condition was mildly increased. These differences with RNA-seq
498 analysis may be a result of one SlyA binding site in the promoter region *sopD*. DNA-
499 protein interaction was also observed at a SlyA concentration of 6.06 nM (Fig. 4A). For
500 *sopE2*, DNA-protein binding occurred at a SlyA concentration of 12.12 nM, indicating the
501 weakest interaction of these promoters (Fig. 4B). Under the *hilA* promoter, reporter activity
502 in Δ*slyA* was increased under oxidative stress (Fig. 4C). DNA-protein interaction was
503 observed at a SlyA concentration of 3.03 nM (Fig. 4C). These results suggest that SlyA
504 negatively regulates *hilA*.

505 Reporter activity under the *fruK* promoter in all conditions was significantly lower
506 in the Δ*slyA* strain compared to WT. In the EMSA, the *fruK* promoter interacted with the

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SlyA protein at a concentration 3.03 nM. SlyA, thus, seems to upregulate *fruK* expression by directly binding to the promoter region (Fig. 4D). Under the *glpA* promoter, reporter activity was slightly lower under oxidative stress in the mutant than in the WT strain. EMSA results showed an interaction between the *glpA* promoter and SlyA at a concentration of 3.03 nM (Fig. 4E). These results suggest that SlyA upregulates the *glpA* gene. The *kgtP* promoter activity was higher in Δ*slyA* than in WT under all conditions, but this difference was more significant under control conditions than under oxidative stress. SlyA interaction with the promoter region of *kgtP* starts at 3.03 nM SlyA (Fig. 4F). SlyA thus seems to downregulate *kgtP* expression by direct binding to the promoter region. Finally, under the *pagC* promoter, reporter activity in control condition was significantly lower in the Δ*slyA* strain compared to WT, and in response to NaOCl, the activity was slightly lower in Δ*slyA* than the WT strain. SlyA interaction with the promoter region of *pagC* occurred at a SlyA concentration of 9.09 nM (Fig. 4G), suggesting that SlyA positively and directly regulates *pagC* expression, which is similar to previous findings [7, 13,14].

3.6 Metabolism and virulence are regulated by SlyA

To effectively evaluate whether SlyA was involved in the regulation of metabolic pathways and virulence, we performed different biochemical assays using various pathways that are affected in the oxidative stress condition. First, we evaluated total thiol quantification (Fig. 5A) because we found that SlyA positively regulates a putative cysteine synthase (STM14_0542). Under control conditions, total thiols were found to amount to 40 mmol/g in both strains. Under H_2O_2 stress, there was a significant decrease in reduced thiols (RSH) in Δ*slyA* compared to WT, where total thiols were increased to 60 mmol/g.

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These results are consistent with the RNA-seq analysis, where the absence of SlyA should decrease the amount of cysteines available for thiol formation. Under NaOCl stress, thiol levels decreased to approximately 38 mmol/g in both strains, possibly because the main damaging effect of NaOCl is chlorination. These results suggest that SlyA is important for maintaining the thiol oxidation rate in the presence of H_2O_2.

To determinate if central metabolism is regulated by SlyA, we measured the pyruvate and NAD^+ in the strains study. Consistent with KEGG database information, where the pyruvate pathway is negatively regulated by SlyA under H_2O_2 conditions, the amount of pyruvate kinase was significantly higher in Δ*slyA* under this condition. Under NaOCl treatment conditions, the amount of pyruvate kinase was also significantly higher compared to WT, although to a lesser degree than under H_2O_2 conditions (Fig. 5B). The pyruvate pathway is inhibited by SlyA, and there are two important pathways that could be compensating and potentiating bacterial central metabolism. These are the glycolysis pathway and the pentose phosphate pathway, which are positively regulated by SlyA. To effectively determine whether central metabolism is induced by SlyA, we measured NAD^+ in both strains under all conditions, and we observed that in Δ*slyA*, NAD^+ levels were significantly lower in control and H_2O_2 conditions (Fig. 5C), suggesting that SlyA positively regulates central metabolism.

We next focused on the role that SlyA plays in modulating *Salmonella* virulence traits. The ability of strains to bind Congo red from the culture medium correlates well with virulence properties because of the presence of a type-III secretion system [24,35]. Under both control and H_2O_2 conditions, Congo red binding was not significantly different in Δ*slyA* and WT. This was in contrast to NaOCl-induced stress, where the Congo red binding

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activity was significantly higher in Δ*slyA* than WT (Fig. 5D). These results are consistent with the RNA-seq analysis, in which the expression levels of certain genes that are part of the type-III secretion system were negatively regulated by SlyA. To determine if SlyA effectively regulates these genes, we measured transcript levels of bacterial *sopD* and *sopE2* (STm14028s, Δ*slyA* and Δ*slyA*/pBR::*slyA* strains) that were recovered from macrophages 1 and 3 h post-infection. Our results showed that at 3 h post-infection only, *sopD* and *sopE2* were negatively regulated by SlyA (Fig. 5E), with Δ*slyA* expression increased two- and five-times, respectively. These results are conclusive because the Δ*slyA* strain entered fewer to macrophages than the WT strain (Supplementary Fig. 2). Additionally, the Δ*slyA*/pBR::*slyA* strain results showed that at 1 h post-infection, the transcript-level behaviour is similar to other strains, but 3 h post-infection, the Δ*slyA*/pBR::*slyA* strain was similar to WT strain, suggesting that SlyA regulates these genes under infectious conditions.

Finally, we measured the percentage of biofilm formation and the importance of SlyA in this process. Bacterial biofilm is involved in cellular communication processes and offers protection from adverse environmental conditions, such as oxidative stress, thereby contributing to a successful host colonisation [36]. We found that biofilm formation percentages were increased in Δ*slyA*, reaching statistical significance under conditions of oxidative stress (Fig. 5F). These results are consistent with the RNA-seq analysis because both experimental approaches point towards a deregulation of outer membrane proteins in the presence of both H_2O_2 and NaOCl (Supplementary Tables 2 and 3), and outer membrane proteins seem to be mostly negatively regulated by SlyA. Thus, biofilm formation would increase as a compensatory mechanism to protect the bacteria against oxidative stress.

578

579 *3.7 SlyA is required for an efficient response to H_2O_2 and NaOCl*

580 To determine whether SlyA is required for the response to H_2O_2 and NaOCl, we
581 evaluated protein carbonylation and membrane lipid peroxidation as indicators of oxidative
582 damage. Treatment with H_2O_2 and NaOCl did not increase carbonylation levels in the WT,
583 whereas in the Δ*slyA* strain, carbonylation levels increased by 50% in the presence of H_2O_2
584 and by 80% in the presence of NaOCl (Fig. 6A).

585 Lipid peroxidation levels, expressed as pmol TBARs/mg of protein, were increased
586 10-fold in WT bacteria exposed to oxidative conditions compared to control conditions. In
587 the Δ*slyA* strain, lipid peroxidation was increased approximately 30 times after exposure to
588 oxidative stress compared to untreated controls, and increased three times compared to WT
589 under the same conditions (Fig. 6B). These results indicate a significantly increased level of
590 oxidative damage in the Δ*slyA* strain after treatment with H_2O_2 and NaOCl, compared to
591 WT under the same conditions.

592 Following these indicators of increased oxidative damage in Δ*slyA* compared to WT
593 after treatment with H_2O_2 and NaOCl, we measured total ROS levels. The only significant
594 increase in ROS levels was observed in the Δ*slyA* strain under exposure to NaOCl, where
595 ROS levels were approximately double those of WT (Fig. 6C). ROS levels do not increase
596 after treatment with H_2O_2, which suggests that bacterial defence mechanisms against
597 oxidative stress include the activation of regulon OxyR, which is involved in H_2O_2
598 tolerance. Consequently, there would be no increase in intracellular ROS [4]. ROS analysis
599 results confirm the RNA-seq analysis results, which showed that in the presence of NaOCl,
600 SlyA upregulates genes involved in the pentose phosphate pathway, including a putative

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601 transketolase and a putative sugar kinase. Increased activity of this pathway, which
602 enhances reducing power, would protect bacteria against ROS.

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4. Discussion

The toxic compounds H_2O_2 and NaOCl have diverse effects on macromolecules [5,37], and when ROS concentrations increase above basal levels, bacteria are damaged in several ways. To evaluate the requirement for SlyA to respond to ROS in *S.* Typhimurium, we assessed oxidative damage indicators, such as protein carbonylation and membrane lipid-peroxidation, which were found to be increased in the Δ*slyA* strain in the presence of 3 mM NaOCl (Fig. 6A and 6B), causing increasing ROS levels. However, in the presence of 1 mM H_2O_2, the OxyR regulon exerts its detoxification function, as described previously (Fig. 6C). Basal levels of both damage markers and total ROS are similar to those of the WT strain under non-stressed conditions, which indicates that SlyA is specifically required for the response to ROS, but not for the maintenance of basal conditions. Additionally, we determined total thiol levels under H_2O_2 oxidative stress, and found a decrease in the Δ*slyA* strain compared to WT (Fig. 5A). Moreover, the RNA-seq transcriptomic analysis showed that SlyA positively regulates a putative cysteine synthase that is expressed after H_2O_2 treatment. Thus, in the absence of this regulator, thiol levels diminish.

Additionally, we determined from whole transcriptomic analysis that the gene encoding the 1-phosphofrucktokinase (*fruK*) is positively regulated by SlyA. To be used, fructose must enter the cell through the mannose permease (ManXYZ) [38], which is also positively regulated by SlyA. Together, the upregulation of these genes would favour glucose formation and entry into the cell. Diverse genes coding for enzymes involved mainly in the productive phase of glycolysis are also positively regulated by SlyA (Supplementary Tables, Fig. 3D and 4D). *S.* Typhimurium, thus, promotes the glycolytic pathway in response to H_2O_2. This is consistent with a previous report by Rui *et al*. [39], which found that in *E. coli*, O_2^- treatment induces ATP gaining-phase enzymes. An increase

in glycolysis would lead to a rise in NADH levels, which would in turn contribute to the response to H_2O_2 through its antioxidant properties. NADH antioxidant properties were suggested to play a role in ROS resistance in *Salmonella*, and in ATP generation through glycolysis. Enhanced ATP generation would allow bacteria to perform processes such as the conversion of NADH to NADPH via NAD kinase, leading to a more efficient response to ROS [40]. Our results suggest that SlyA contributes to metabolism regulation by accumulation of NAD^+ under oxidative stress (Fig. 5C), which is consistent with previous results.

In *S.* Typhimurium, it has been established that SlyA modulates SPI-2 expression via its regulation of the kinase sensor SsrA, which is involved in SPI-2 expression through binding to the promoter region [8]. Because of our study conditions, we did not observe genes belonging to SPI-2, which are expressed mainly under low Mg^{+2} or acidic conditions. However, we found the *ydgT* (STM14_1760) gene that encodes a nucleotide-like protein, a negative regulator that represses SPI-2 transcription [41], and that is negatively regulated by SlyA under H_2O_2 conditions. Additionally, SlyA can reverse the inhibition of the negative regulator H-NS, which acts on different genes [13,14]. This suggests that SlyA regulates SPI-2 through both SsrA activation and inhibition of its negative regulator. Results from transcriptome analysis indicate that SlyA negatively regulates SPI-1 genes, which is consistent with a report by Colgan [42], where SPI-1 gene expression increased in absence of *slyA*. Thus, it is possible that through a signal encountered in the intestine, SlyA might modulate its response to toxic compounds, such as H2O2 or OCl^-, repressing genes involved in the cellular invasion (*invF, hilA* y *spaR*) and effector proteins (*sopD* y *sopE2*) that are required for eukaryotic cell invasion [43–45]. This is consistent with previous reports on HilA, a global regulator required for SPI-1 gene expression [46, 47].

Additionally, HilA can bind to specific sequences in the promoter of *invF*, a transcriptional regulator, which is necessary for the expression of a sub-group of genes related to invasion [48]. Previous research showed that an increase in *hilA* expression causes a survival defect, suggesting the harmful effects of not repressing SPI-1 during invasion [49]. In summary, the expression of genes encoding effector and invasion-related proteins is significantly increased in the Δ*slyA* strain (Fig. 3), suggesting negative regulation by SlyA. We can infer that this negative regulation is a consequence of SlyA binding to the *hilA* promoter region (Fig. 4C), which affects the expression of all HilA-regulated genes in a downstream cascade. Additionally, cytotoxicity in macrophages is induced by SPI-1 TTSS [50], which changes the activation state of macrophages, and there is, therefore, a decrease in salmonella virulence, where SlyA could play a key role in the infective process by repressing the expression of effector proteins belonging to the TTSS. Finally, our Congo red binding assays provided an indirect measure of TTSS activity and it was slightly increased in Δ*slyA* strains compared to WT, indicating the presence of TTSS and a likely link to the strain's virulence properties (Fig. 5D). The transcription levels of *sopD* and *sopE2* under infection conditions also showed a remarkable increase 3 h post-infection (Fig. 5D), and these effects were observable even when the Δ*slyA* strain was less invasive (Supplementary Fig, 2), suggesting that SlyA negatively regulates these genes.

Finally, we evaluated biofilm formation as an important part of bacterial metabolism and a protection against a hostile environment inside the host. We found that the *csgD* gene that encodes one of the master regulators involved in biofilm formation, CsgD [51], which is negatively regulated by SlyA under NaOCl stress. These results are consistent with results showing that biofilm formation is increased in the Δ*slyA* under all conditions evaluated (Fig. 5F). Additionally, the capacity for biofilm formation by

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675 *Salmonella* spp. depends on the surface and the resistance to treatment, and lower
676 concentrations of chloride (50 ppm) were found to partially inactivate biofilm formation. At
677 100 ppm chloride, no formation was detected [52].

678 In conclusion, SlyA can regulate different metabolic pathways, from central
679 metabolism to oxidative stress defence through the activation of alternative pathways, and
680 thereby enhancing bacterial reduction potential. These data reveal the mechanisms by
681 which SlyA might modulate the course of *S.* Typhimurium infection. Our findings
682 contribute to an improved understanding of *Salmonella* physiology in response to oxidative
683 stress that is encountered during the host immune defence.

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833 **Legends to figures**

834 **Figure 1. Functional classification of the differentially expressed genes in the**
835 ***Salmonella* Typhimurium Δ*slyA* as compared to the WT in response to H_2O_2 and**
836 **NaOCl.** The bars represent the percentage of genes belonging to each group that were
837 altered in the mutant strain exposed to **A)** H_2O_2 1 mM and **B)** NaOCl 3 mM. The pathways
838 have been defined by KEGG data base (*Salmonella enterica* serovar Typhimurium 14028s
839 KEGG) using the data of a RNA-seq of the mutant to search each pathway and the
840 percentage was calculated using the total of genes that was altered in the mutant strain. The
841 red bars represent all the genes that has a negative regulation by SlyA and the blue bars a
842 positive regulation by SlyA. The percentage of genes represents the total number of genes
843 differentially expressed in the RNA-seq assay.

844

845 **Figure 2. Comparative transcriptomic patterns between *S.* Typhimurium WT and**
846 **Δ*SlyA* strains in response to ROS.** Transcripts whose expression levels differed between
847 *S.* Typhimurium WT and transcription factor SlyA mutant strains after 20 min treatment
848 with **A)** 1 mM H_2O_2 and **B)** 3 mM NaOCl. For each gene, the log2 of the fold-change of
849 expression is shown (FDR ≤ 0.1), according to the heat-scale in the bottom right corner.

850

851 **Figure 3. Validation of RNA-seq results by qRT-PCR of strains STm 14028s, Δ*slyA***
852 **and Δ*slyA*/pBR::*slyA* exposed to H_2O_2 or NaOCl.** Panels show expression levels of the
853 mutant and complemented strains relative to the WT, in response to H_2O_2 (1 mM) or
854 NaOCl (3 mM), of **A)** *sopD*, **B)** *sopE2*, **C)** *hilA*, **D)** *fruK*, **E)** *glpA*, **F)** *kgtP* and **G)** *pagC*.
855 All data were normalized against *talB*. Values represent the average of five independent
856 experiments ± SD (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

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857

858 **Figure 4. Direct regulation of *sopD, sopE2, hilA, fruK, glpA, kgtP* and *pagC* genes by**
859 **SlyA in response to ROS.** Panel **I**, promoter reporter activity in response to H_2O_2 (1 mM)
860 or NaOCl (3 mM); panel **II**, electrophoretic mobility shift assay (EMSA) of *sopD*, *sopE2*,
861 *hilA*, *fruK*, *glpA*, *kgtP* and *pagC* promoter regions after incubation with increasing amount
862 of SlyA (all fragments were 300 bp in length and a 120 bp fragment of the *ompX* promoter
863 was used as a negative control). Results are shown in **A)** *sopD*, **B)** *sopE2*, **C)** *hilA*, **D)** *fruK*,
864 **E)** *glpA*, **F)** *kgtP* and **G)** *pagC*. Values represent the average of five independent
865 experiments ± SD (*p < 0.05, **p < 0.01, ***p < 0.001).

866

867 **Figure 5. Validation of metabolic pathways regulated by SlyA in response to H_2O_2 (1**
868 **mM) and NaOCl (3 mM). A)** Total thiols formation **B)** Pyruvate kinase activity **C)** NAD^+
869 levels **D)** Congo red binding assay **E)** Relative expression of *sopD* and *sopE2* genes in
870 STm14028s, Δ*slyA* and Δ*slyA*/pBR::*slyA* strains recovered from macrophages at 1 and 3 h
871 post-infection. **F)** Percentage of biofilm formation in *Salmonella* strains STm14028s and
872 Δ*slyA* exposed to H_2O_2 and NaOCl. Metabolic activity in the WT strain was taken as 100%.
873 Values represent the average of seven independent experiments ± SD (*p < 0.05, **p <
874 0.01, ***p < 0.001).

875

876 **Figure 6. Indicators of oxidative damage in *S.* Typhimurium strains STm 14028s and**
877 **Δ*slyA* exposed to H_2O_2 (1 mM) and NaOCl (3 mM). A)** Carbonyl levels (DNPH
878 method), **B)** lipid peroxidation levels (TBARS method), and **C)** intracellular ROS levels in
879 STm14028s and Δ*slyA* strains, all after 20 min exposure to H_2O_2 (1 mM) and NaOCl (3

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mM). Values represent the average of three independent experiments ± SD (*p < 0.05, **p < 0.01, ***

Supplementary figure 1. Comparative transcriptomic patterns of *S.* Typhimurium WT (**A** and **B**) and Δ*SlyA* **(C)** strains under different conditions. Transcripts with altered expression levels in the presence of H_2O_2 (A and C) or NaOCl (B) versus control conditions in *S.* Typhimurium WT and *slyA* mutant strains. For each gene, the log2 of the fold-change of expression is shown (FDR ≤ 0.1), according to the heat-scale in the bottom right corner.

Supplementary figure 2. Relative of infections of mutant and complemented vs WT strains harvested 1 and 3 h post-infection from Raw 264.7 (***p<0.001).

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Table 1. Bacterial strain used in this study

Strain	Relevant characteristic	Source
14028s	Wild type strain *Salmonella enterica* serovar Typhimurium.	Facilitated by Dr. G. Mora ATCC
14028s/pGLO	14028s transformed with empty vector pGLO expressing green fluorescent protein (GFP).	This work
14028s/pGLO::P*fruK*	14028s transformed with vector pGLO carrying *fruk* gene promoter upstream of the reporter gene.	This work
14028s/pGLO::P*sopD*	14028s transformed with vector pGLO carrying *sopD* gene promoter upstream of the reporter gene.	This work
14028s/pGLO::P*glpA*	14028s transformed with vector pGLO carrying *glpA* gene promoter upstream of the reporter gene.	This work
14028s/pGLO::P*kgtP*	14028s transformed with vector pGLO carrying *kgtP* gene promoter upstream of the reporter gene.	This work
14028s/pGLO::P*sopE2*	14028s transformed with vector pGLO carrying *sopE2* gene promoter upstream of the reporter gene.	This work

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14028s/pGLO::P*hilA*	14028s transformed with vector pGLO carrying *hilA* gene promoter upstream of the reporter gene.	This work
14028s/pGLO::P*pagC*	14028s transformed with vector pGLO carrying *pagC* gene promoter upstream of the reporter gene.	This work
Δ*slyA*	*slyA*::*aph*	C. Saavedra
Δ*slyA*/pBR::*slyA*	Δ*slyA* transformed with the vector pBR322 carrying the slyA gene	C. Saavedra
Δ*slyA*/pGLO	Δ*slyA* transformed with empty vector pGLO expressing green fluorescent protein (GFP).	This work
Δ*slyA*/pGLO::P*fruK*	Δ*slyA* transformed with vector pGLO carrying *fruk* gene promoter upstream of the reporter gene.	This work
Δ*slyA*/pGLO::P*sopD*	Δ*slyA* transformed with vector pGLO carrying *sopD* gene promoter upstream of the reporter gene.	This work
Δ*slyA*/pGLO::P*glpA*	Δ*slyA* transformed with vector pGLO carrying *glpA* gene promoter upstream of the reporter gene.	This work
Δ*slyA*/pGLO::P*kgtP*	Δ*slyA* transformed with vector pGLO carrying *kgtP* gene promoter upstream of the reporter gene.	This work

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Δ*slyA*/pGLO::P*sopE2*	Δ*slyA* transformed with vector pGLO carrying *sopE2* gene promoter upstream of the reporter gene.	This work
Δ*slyA*/pGLO::P*hilA*	Δ*slyA* transformed with vector pGLO carrying *hilA* gene promoter upstream of the reporter gene.	This work
Δ*slyA*/pGLO::P*pagC*	Δ*slyA* transformed with vector pGLO carrying *pagC* gene promoter upstream of the reporter gene.	This work
Top10	F- mcrA Δ(mrr-hsdRMS-mcrBC) φ80lacZΔM15 ΔlacX74 nupG recA1 araD139 Δ(ara-leu)7697 galE15 galK16 rpsL(StrR) endA1 λ-.	Invitrogen
BL21(DE3)	F– ompT gal dcm lon hsdSB(rB- mB-) λ(DE3[lacI lacUV5-T7 gene 1 ind1 sam7 nin5]).	Invitrogen
BL21(DE3)/pET-*slyA*	BL21(DE3) transformed with the pET-TOPO101SlyA vector carrying the *S.* Typhimurium *slyA* gene.	This work

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Table 2. Primers used in this study

Gene	Forward primer 5'→3'	Reverse primer 5'→3'
pGLO-*fruK*	GCACCGGTCTGACGCAAGCTGAAAGA	CGCGCTAGCCATTCTCCTGCTGAATTGAAACG
pGLO-*sopD*	GCACCGGTTGCCTTCTGCATAAACCAA	CGCGCTAGCCATGCTTAAAGTGACTGGCATAA
pGLO-*glpA*	GCACCGGTATTGTAGCCTCCGTGGC	CGCGCTAGCCATTGTTTTTCCTCACAGTTCG
pGLO-*kgtP*	GCACCGGTAATACACGGGGTAATGATCC	CGCGCTAGCCATACTGTTCTCCTTGTTGCCA
pGLO-*sopE2*	GCACCGGTACGCAGTAGTTGAATTGAAG	CGCGCTAGCCATGGTAGTTCTCCTTTTAGATA
pGLO-*hilA*	GCACCGGTAATCACAGTTAGTTATAACAATA	CGCGCTAGCCATTCTCTTACAGGGTGAAAAGT
pGLO-*pagC*	GCACCGGTGCGTGAGAAAAATTAGCATTC	CGCGCTAGCCATCTACTTATTATTTACGGTGTGTT
pGLO_cheq	GTAGTGATGAATCTCTCCTG	CCTTCACCCTCTCCACTGAC
pET_*slyA*	CACCATGAAATTGGAATCGCCACT	ATCGTGAGAGTGCAATTCCA
qRT-*fruK*	AACGCTTTGTTAACGACTCC	TCACGGCTACTATCAAAGAT
qRT-	GATGGACGCTTCTCAGAC	TACTCATAGATTCTATCACTG

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sopD		C
qRT-*glpA*	CAATATGTTGGATGCCAGAG	TTCGCCAGTGAGATGGTT
qRT-*kgtP*	TATGAGTGAAGTTGCGCTTG	AATCACAACGACCAGCAATG
qRT-*sopE2*	TTTTCATCGTGGGAACGCTT	AATATGGCTTCGCATGTCTG
qRT-*hilA*	GTGAAGGGATTATCGCAGTA	CGTAATTGATCCATGAGCTC
qRT-*pagC*	CGAGGGGTAAATGTGAAATA	ACCGTACTTCACCTCAAACT
qRT-*talB*	ACTATGCGCCAGCTGAAGAT	TCGCTTTCCGCCAGTTCTTT

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Table 3. Candidate SlyA binding sites from promoter region of genes evaluated
Sequences shown are those with the greatest similarity to the determined consensus sequence 5'-TTWGBAWTBWAA-3' (B = C, G or T; W = A or T) [6].

Promoter Region	Possible Binding Site	Location relative to start codon
p*sopD*	TTATCTGTTTAA	93 pb upstream of *sopD*
p*sopE2*	TTATAAATATCA ATAGTTATCTAA	96 pb upstream of *sopE2* 13 pb upstream of *sopE2*
p*hilA*	TTAGTTATAACA TTAGTACTAAGA TTAGTTATTATA	225 pb upstream of *hilA* 83 pb upstream of *hilA* 33 pb upstream of *hilA*
p*fruK*	TTTGATAACTCA TTTCAATTCAGC	60 pb upstream of *fruK* 14 pb upstream of *fruK*
p*glpA*	TTATGCGCGAAA TTTGTATGGCTA	60 pb upstream of *glpA* 31 pb upstream of *glpA*
p*kgtP*	TTAGCAAACAAA	85 pb upstream of *kgtP*
p*pagC*	TTAGCATTCAAA CTTCGGTAGTAA CTAGTATTAAGG	219 pb upstream of *pagC* 152 pb upstream of *pagC* 7 pb upstream of *pagC*

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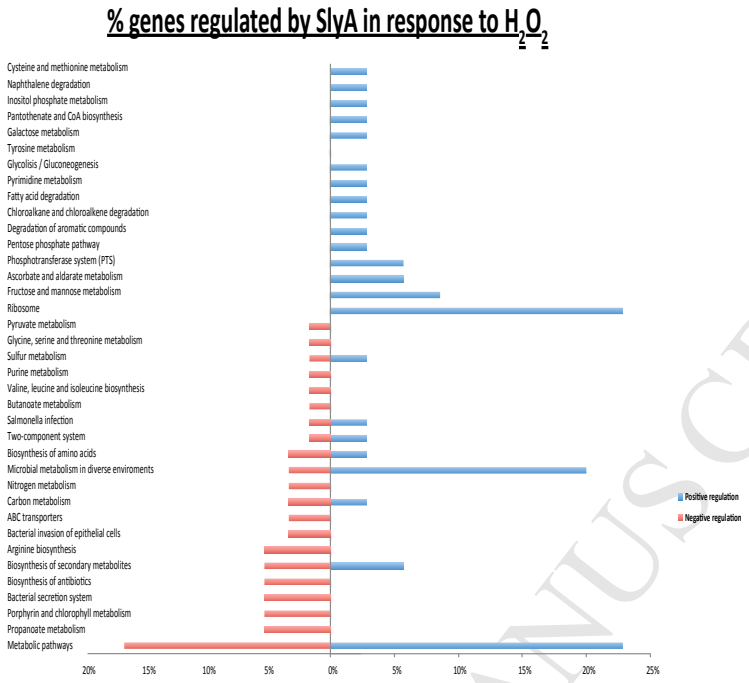

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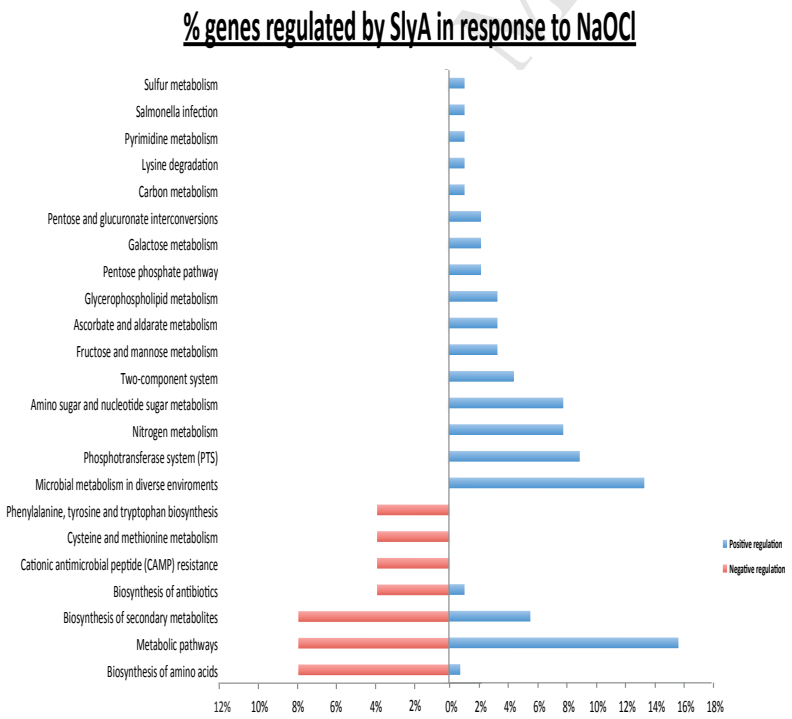

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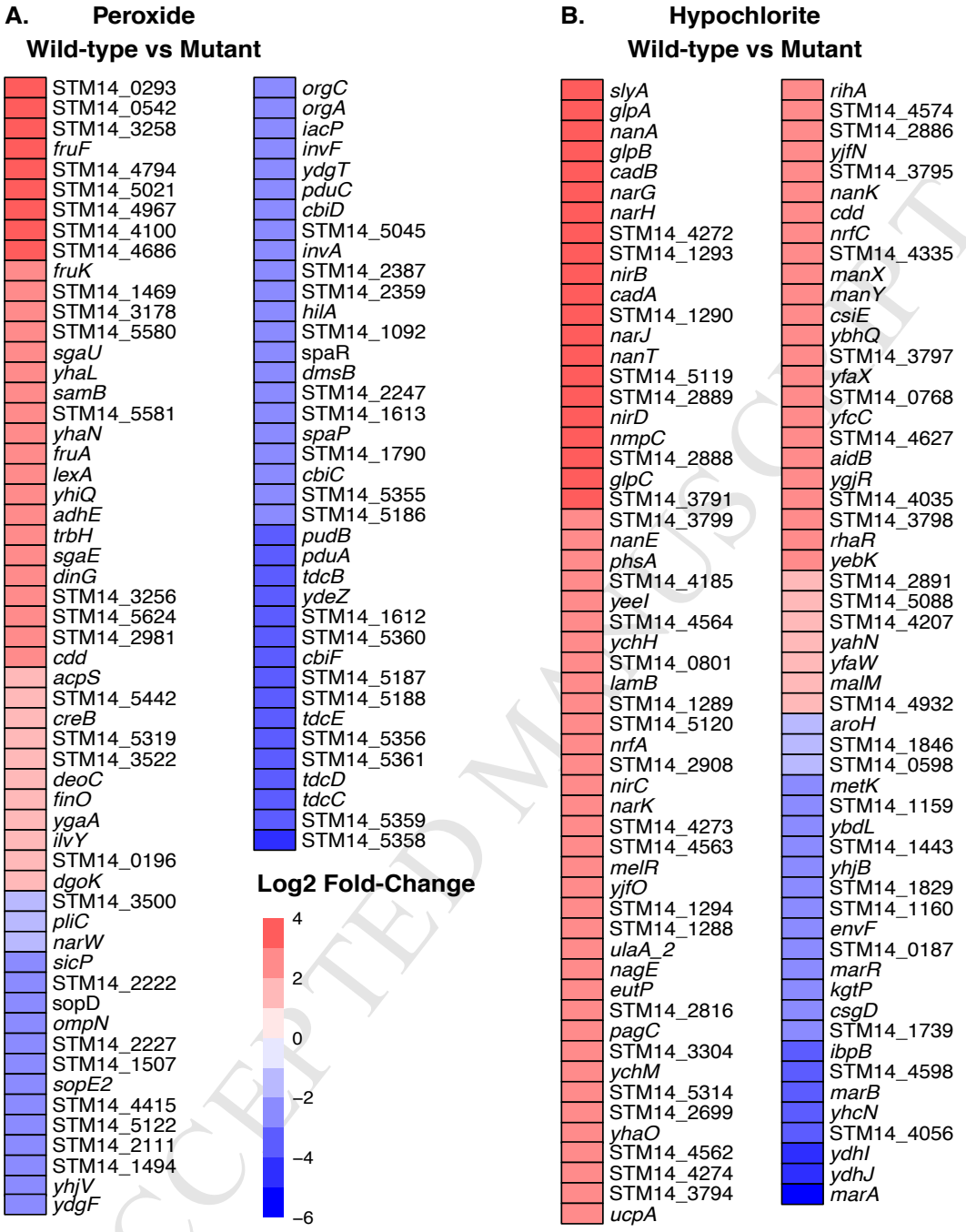

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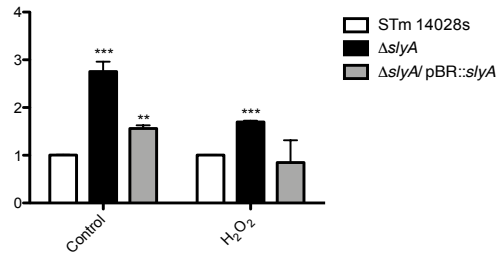

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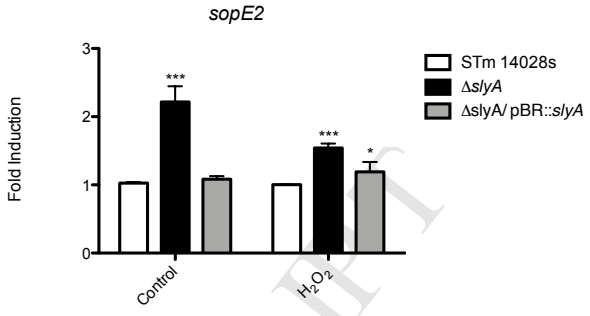

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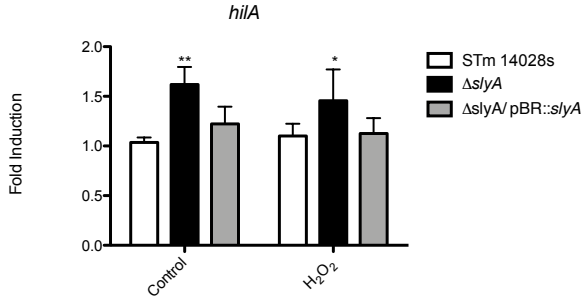

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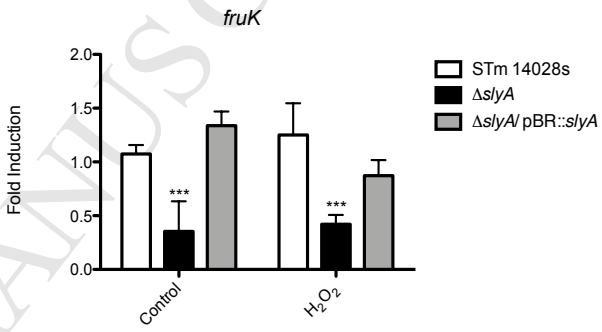

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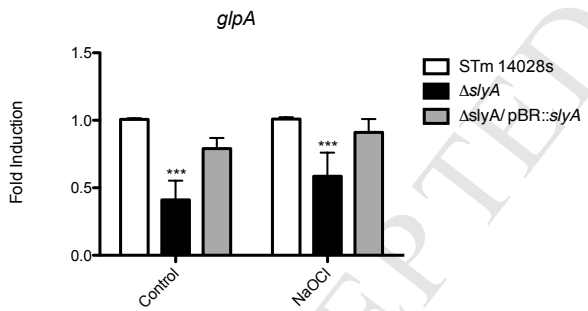

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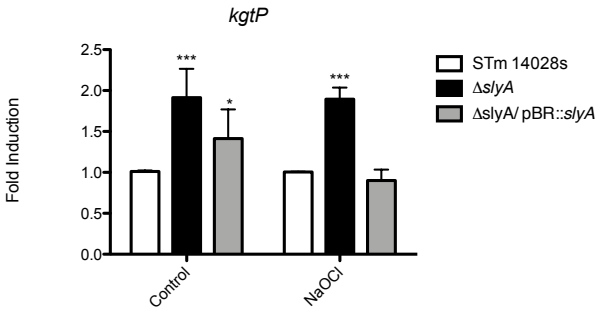

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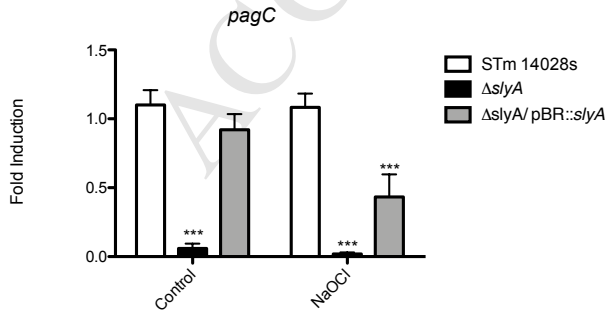

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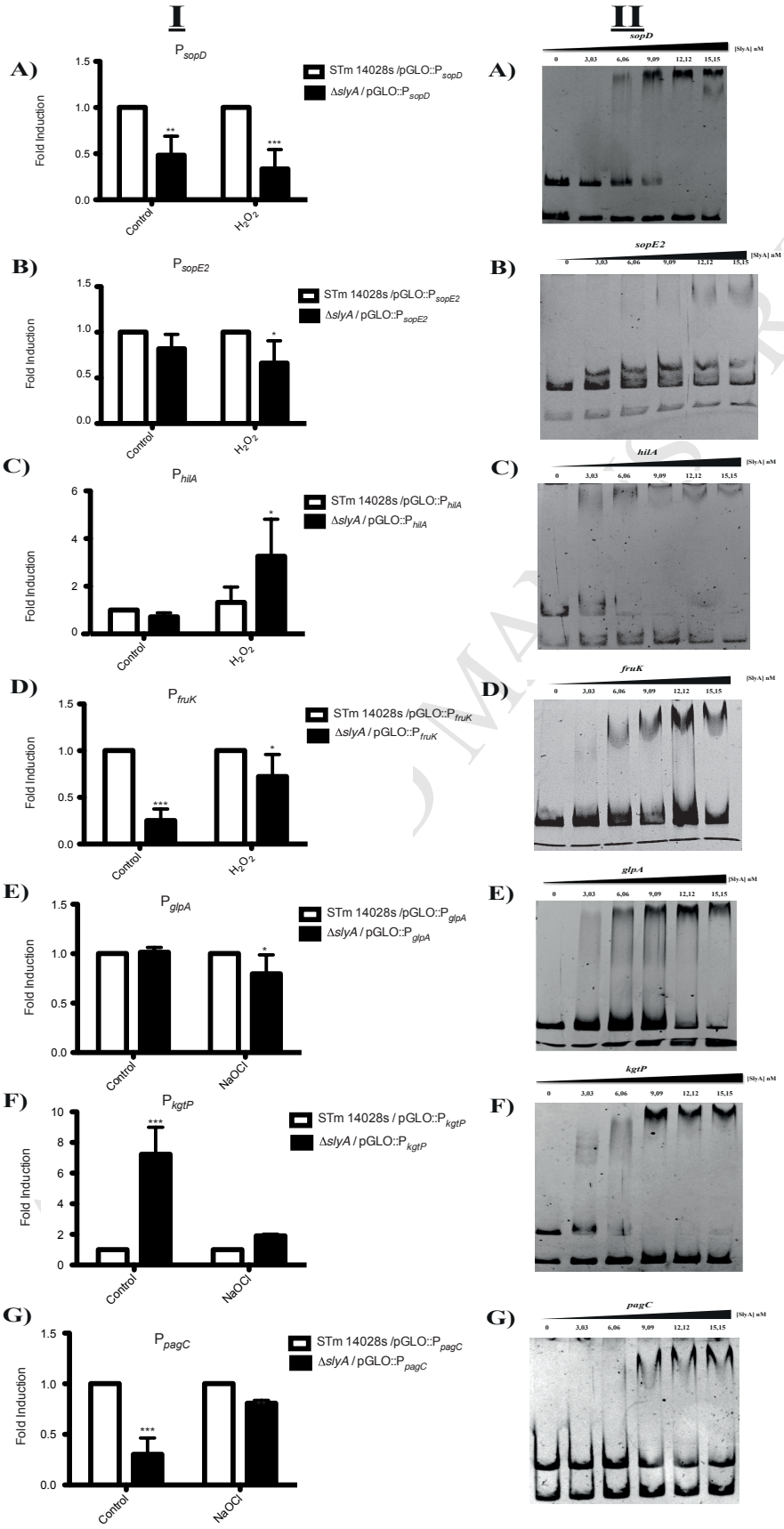
I
II
A)
P sopD
STm 14028s /pGLO::P sopD
ΔslyA / pGLO::P sopD
Fold Induction
Control
H2O2
sopD
[SlyA] nM
B)
P sopE2
STm 14028s /pGLO::P sopE2
ΔslyA / pGLO::P sopE2
sopE2
C)
P hilA
STm 14028s /pGLO::P hilA
ΔslyA / pGLO::P hilA
hilA
D)
P fruK
STm 14028s /pGLO::P fruK
ΔslyA / pGLO::P fruK
fruK
E)
P glpA
STm 14028s /pGLO::P glpA
ΔslyA / pGLO::P glpA
NaOCl
glpA
F)
P kgtP
STm 14028s / pGLO::P kgtP
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G)
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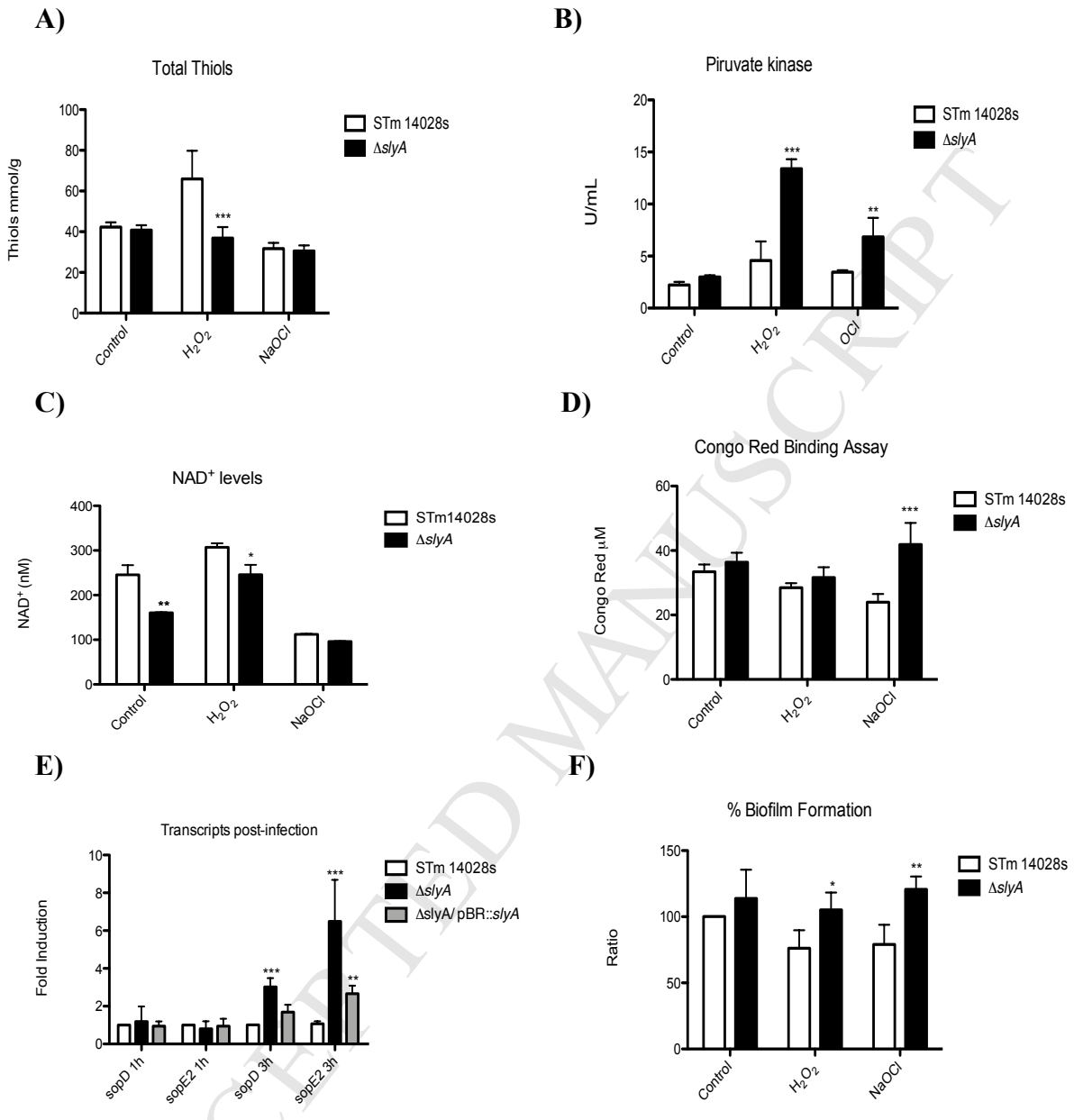
pagC



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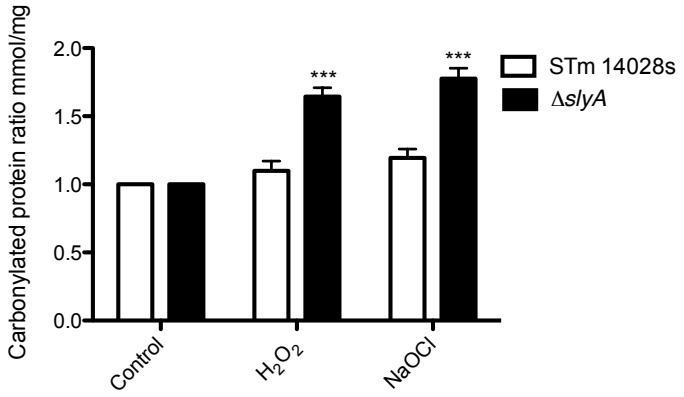

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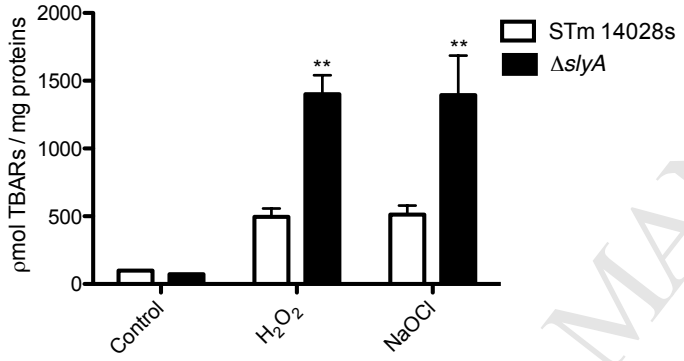

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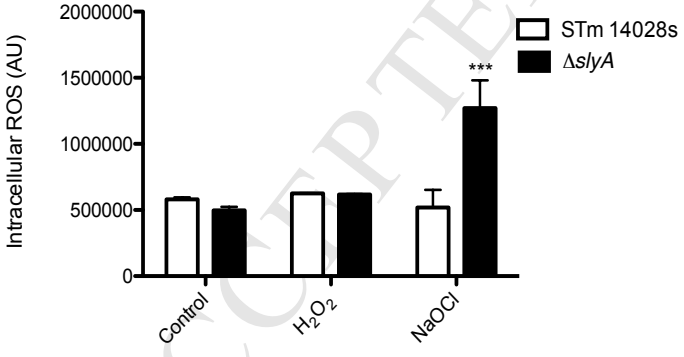

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