Probing the ArcA regulon under aerobic/ROS conditions in Salmonella enterica serovar Typhimurium

Eduardo H Morales1,3,4†, Bernardo Collao1†, Prerak T Desai2, Iván L Calderón1, Fernando Gil1, Roberto Luraschi1, Steffen Porwollik2, Michael McClelland2 and Claudia P Saavedra1*

Abstract
Background: Hydrogen peroxide (H2O2) is a reactive oxygen species (ROS), which is part of the oxidative burst encountered upon internalization of Salmonella enterica serovar Typhimurium (S. Typhimurium) by phagocytic cells. It has previously been established that the ArcAB two-component system plays a critical role in ROS resistance, but the genes regulated by the system remained undetermined to date. We therefore investigated the ArcA regulon in aerobically growing S. Typhimurium before and after exposure to H2O2 by querying gene expression and other physiological changes in wild type and ΔarcA strains.

Results: In the ΔarcA strain, expression of 292 genes showed direct or indirect regulation by ArcA in response to H2O2, of which 141 were also regulated in aerobiosis, but in the opposite direction. Gene set enrichment analysis (GSEA) of the expression data from WT and ΔarcA strains, revealed that, in response to H2O2 challenge in aerobically grown cells, ArcA down regulated multiple PEP-PTS and ABC transporters, while up regulating genes involved in glutathione and glycerolipid metabolism and nucleotide transport. Further biochemical analysis guided by GSEA results showed that deletion of arcA during aerobic growth lead to increased reactive oxygen species (ROS) production which was concomitant with an increased NADH/NAD+ ratio. In absence of ArcA under aerobic conditions, H2O2 exposure resulted in lower levels of glutathione reductase activity, leading to a decreased GSH (reduced glutathione)/GSSG (oxidized glutathione) ratio.

Conclusion: The ArcA regulon was defined in 2 conditions, aerobic growth and the combination of peroxide treatment and aerobic growth in S. Typhimurium. ArcA coordinates a response that involves multiple aspects of the carbon flux through central metabolism, which ultimately modulates the reducing potential of the cell.

Keywords: ArcAB two-component system, Oxidative stress, Hydrogen peroxide resistance

Background
Salmonella enterica serovar Typhimurium (S. Typhimurium) is a Gram-negative, facultative anaerobe and intracellular bacterium that causes gastroenteritis, bacteremia and enteric fever in the murine model [1]. During its infective cycle, S. Typhimurium is internalized by phagocytes where it is exposed to a series of antimicrobial compounds including reactive oxygen species (ROS) which trigger the production of superoxide (O2•−) by phagocytic NADPH oxidase. O2•− is unstable with a half life in the order of milliseconds, and under acidic conditions, as those found within the Salmonella containing vacuole (SCV), two molecules of O2•− react to generate H2O2 [2]. Additionally, the S. Typhimurium genome codes for both cytoplasmic (sodA and sodB) and periplasmic (sodCI and sodCII) superoxide dismutases, that catalyze the generation of H2O2 and molecular oxygen from O2•− [3-5].

The response of the bacterium to H2O2 has been mostly related to the transcription factor OxyR [6], however, several studies in Escherichia coli (E. coli), S. Typhimurium, S. Enteritidis and Haemophilus influenzae indicate that the response regulator ArcA is required for...
H$_2$O$_2$ resistance [7-10]. Furthermore, in *E. coli* H$_2$O$_2$ resistance depends on the cognate sensor ArcB [7]. The ArcAB two-component system is composed of the response regulator ArcA and the hybrid sensor kinase ArcB [11,12]. ArcAB responds to shifts in oxygen concentration [13,14], however, the signal that activates the system remains elusive since some studies show no correlation between ArcAB activity and the ubiquinone pool [13], while others indicate that the system’s activity depends on the ubiquinone and/or menaquinone pools [15-17]. Upon reduction of the redox-active cysteine residues between two monomers, ArcB undergoes autophosphorylation in an ATP-dependent intramolecular reaction at a conserved histidine residue located at position 292 [18,19]. The signal is transferred to residues D576 and H717 of ArcB and finally to residue D54 of ArcA [12,19,20]. Phosphorylated ArcA (ArcA-P) forms a tetramer of dimers in a 1:1 ratio of ArcA and ArcA-P, which binds to promoter regions, thereby regulating gene expression [21,22].

Several studies in *E. coli*, *S. Typhimurium*, *H. influenzae* and *Shewanella oneidensis* have used global gene expression profiling to determine the ArcA regulon under anaerobic conditions, showing that the effect of ArcA is pleiotropic and coordinates a response that includes changes in cellular metabolism, motility and chromosomal replication, among others [8,10,23-26]. In *S. Typhimurium* 14028s grown under anaerobic conditions, ArcA regulates either directly or indirectly more than 392 genes. Additionally, an *arcA* mutant has a longer doubling time than the wild type strain, lacks flagella, is non-motile and remains fully virulent [8].

In contrast to the vast amount of information about the role of ArcA in anaerobiosis, little is known about the genes or biochemical processes that ArcA regulates in response to H$_2$O$_2$. Previous studies have mainly shown that *arcA* or *arcB* mutant strains are more sensitive to the toxic compound [8-10]. One study conducted in *E. coli* used a proteomic approach and determined that ArcA regulates the expression of *flIC*, *oppA* and *gltI* in response to H$_2$O$_2$ [7], while in *S. Typhimurium* ArcA negatively regulates *ompD* and *ompW* [27,28]. To gain further insights into the role of ArcA in ROS resistance, we compared transcriptional changes in *S. Typhimurium* 14028s wild-type and Δ*arcA* strains with and without peroxide exposure under aerobic conditions. As expected, the Δ*arcA* mutation affected multiple pathways confirming that ArcA has a pleiotropic effect and plays a role as a global regulator. Interestingly, the genes regulated by ArcA in response to H$_2$O$_2$ differ from those regulated under anaerobic conditions [8]. A Gene Set Enrichment Analyses using the KEGG database predicted that 10 pathways were up-regulated and 2 down-regulated by ArcA in response to H$_2$O$_2$ treatment in aerobiosis. Finally, biochemical analyses showed that under aerobic conditions ArcA modulates the redox potential of the cell by regulating the levels of NADH and of intracellular ROS. After H$_2$O$_2$ exposure under aerobic conditions, ArcA was found to regulate turnover of reduced glutathione (GSH).

**Methods**

**Bacterial strains and growth conditions**

Pre-cultures of strains 14028s wild type and Δ*arcA* were streaked from cryo-vials stored at ~80°C onto LB agar plates and allowed to grow at 37°C for 12 h. One colony was picked and grown in a 250 ml Erlenmeyer flask containing 25 ml of LB broth for 16 h at 37°C on a rotary shaker at 200 rpm. Exactly 500 μl of the cultures were then transferred into 500 ml Erlenmeyer flasks containing 50 ml of LB broth and grown in a temperature controlled rotary shaker at 200 rpm (LSI-3016R, Labtech Shaking Incubator, Indonesia). Optical density (OD$_{600}$) was measured until reaching the desired OD$_{600}$ for treatment with H$_2$O$_2$ (~ 0.4, corresponding to an incubation time of about 2.5 h). These conditions closely resemble those used in a previous study with *E. coli*, where 50 ml of culture grown at 200 rpm in a shaking incubator at 37°C to an OD$_{546}$ of ~ 0.4 exhibited a pO$_2$ of ≥ 90% [29]. Solid media contained agar (20 g l$^{-1}$), and plates were incubated at 37°C. When necessary, growth media was supplemented with the appropriate antibiotics.

**Microarray analysis**

Overnight cultures of strains 14028s and Δ*arcA* were diluted (1:100) and cells were grown to OD$_{600}$ ~ 0.4 as described. At this point, H$_2$O$_2$ (1 mM) was added and cells were grown for 20 min. Control cells received no treatment. Experiments were performed in triplicate on different days. After exposure to the toxic compound, 5 ml of ice cold 5% (v/v) phenol pH 4.3 / 95% (v/v) ethanol was added to 25 ml of culture and left on ice for 20 min. Subsequently, 8 ml of this solution were centrifuged for 10 min at 8000 rpm, the supernatant was removed and the bacterial pellet was resuspended with 200 μl of 10 mM Tris–HCl (pH 8.0) that included 4 μl of lysozyme (50 mg/ml). The reaction was incubated for 10 min at 37°C, and total RNA was extracted using the High Pure RNA Isolation kit (Roche) following the manufacturer’s instructions. RNA was eluted in 105 μl of water and treated with DNaseI (Roche) at 37°C for 30 min. Total RNA was recovered using the Qiagen RNeasy kit (Qiagen), following the manufacturer’s instructions. RNA was eluted in 80 μl and subjected to a second round of DNaseI treatment (Ambion Turbo DNA-free kit) at 37°C for 30 min, purified, recovered using the Qiagen RNeasy kit (Qiagen) following the manufacturer’s instructions and eluted in 55 μl of water.
Exactly 20 μg of total RNA were used for labeling with Cy3 or Cy5. Briefly, the RNA volume was adjusted to 30 μl, 2 μl of random hexamers N6 (Sigma, 2 μg/μl) were added and the mixture was incubated for 10 min at 70°C. Subsequently, cDNA was generated using Superscript II (Invitrogen) following the manufacturer’s instructions. Final nucleotide concentrations of the reaction were 0.5 mM dATP, dTTP, dGTP and 0.2 mM dCTP. After addition of the master mix, 4 μl of 5 mM dye labeled dCTP (Cy3 or Cy5) were added to the reaction and the mixture was incubated at 42°C for 60 min. After this time, 2 μl of Superscript II were added and the reaction was incubated at 42°C for an additional 60 min. The reaction was stopped by adding 3 μl of 1 M NaOH and incubating at 70°C for 10 min. The pH was neutralized by adding 3 μl of 1 M HCl. The labeled cDNA was purified using the Qiagen PCR purification kit following the manufacturer’s instructions. The purified labeled cDNA (4 μg) was hybridized to a ~387,000 50-mer NimbleGen microarray (Roche NimbleGen), tiling the S. Typhimurium 14028s genome at overlapping intervals of about 12 bases on both strands, as previously described [30].

Data acquisition and analysis

Arrays were scanned using a GenePix 4000B laser scanner (Molecular Devices, Sunnyvale, California) at 5 μm resolution. Signal intensities were quantified using NimbleScan software v2.4 (Roche NimbleGen). Intensity values were background subtracted, normalized within (median) and between (quantile) the arrays using WebarrayDB [31], and converted to log2 values. For each array, the background was calculated as follows: log 2 median intensity value for negative control probes + (3 * log2 intensity value standard deviation negative control probes). Negative control probes correspond to the probes located on the non-coding strand of each gene in the array. Genes with intensity values over the background were included in the analysis. After array data acquisition and normalization, two-way ANOVA was performed using MeV TM4 software [32], to determine uncorrected p-values. For the analysis, two categories were considered (genotype and treatment), each with two sub-categories. False Discovery Rate (FDR) adjusted q values were calculated using QVALUE in Bioconductor [33]. Genes with a q value ≤ 0.05 for interaction and a ratio of ≥2 between the fold change of strains 14028s wild type and ΔarcA ((wild type treated/wild type control)/(ΔarcA treated/ ΔarcA control)) were considered to be differentially regulated in response to H2O2. Genes with a q-value of ≤ 0.05 for genotype and a fold change of ≥2 between strains ΔarcA and wild type (ΔarcA control/wild type control) without treatment were considered to be differentially regulated in aerobiosis. The microarray data has been deposited in GEO (http://www.ncbi.nlm.nih.gov/geo/) and is accessible via GEO Accession Number GSE34134.

Prediction of metabolic pathways altered in the different strains by treatment with H2O2 or due to the mutation of arcA was performed using the software Gene Set Enrichment Analysis (GSEA) [34], with the KEGG database for S. Typhimurium LT2 as a reference. Briefly, GSEA is a computational method that determines whether an a priori defined set of genes shows statistically significant, concordant differences between two biological states [34]. To determine the pathways regulated by ArcA in response to H2O2, the log2 values of all replicates were averaged and treated as follows: (log2 14028s wild type H2O2 aerobic - log2 14028s wild type aerobic) - (log2 ΔarcA H2O2 aerobic - log2 ΔarcA aerobic). Positive Normalized Enrichment Score (NES) values represent pathways up-regulated by ArcA, while negative NES values represent pathways negatively regulated upon H2O2 treatment under aerobic conditions. To determine the pathways regulated under aerobic conditions, the log2 values of all replicates of untreated cells were averaged and treated as follows: (log2 ΔarcA aerobic - log2 14028s wild type aerobic). Pathways with an FDR of ≤ 0.25 as determined by GSEA were considered to present significant changes.

Real time quantitative RT-PCR

qRT-PCR was performed using the primers listed in Additional file 1: Table S1 as previously described [28], with a minor modification of the PCR program. Briefly, relative quantification was performed using Brilliant II SYBR Green QPCR Master Reagent Kit and the Mx3000P detection system (Stratagene). 16S rRNA was used for normalization. The reaction mixture was carried out in a final volume of 20 μl containing 1 μl of diluted cDNA (1:1000), 0.24 μl of each primer (120 nM), 10 μl of 2× Master Mix, 0.14 μl of diluted ROX (1:200) and 8.38 μl of H2O. The reaction was performed under the following conditions: 10 min at 95°C followed by 40 cycles of 30 s at 95°C, 30 s at 58°C and 30 s at 72°C. Finally, a melting cycle from 65°C to 95°C was performed 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 normalized with 16S levels according to Pfaffl [35].

Promoter analysis

A positional weight matrix was generated using the ArcA-binding sites predicted in E. coli for which footprinting experiments are available reviewed in [36]. Additionally, the binding sites predicted for members of the ArcA regulon in S. Typhimurium 14028s in anaerobiosis...
were also included [8], as was that of the onpW promoter region, which was shown to be functional [28]. The upstream sequences of the genes regulated by ArcA in response to aerobiosis or H2O2 exposure under aerobic conditions (Additional file 2: Table S2) were retrieved (positions −400 to −1 with respect to the translation start site) from the sequenced and annotated genome of S. Typhimurium 14028s [37]. Promoter regions with less than 20 nt between the translation start site of the ORF under analysis and the end or start of the upstream ORF were not included in the analysis. Binding sites at the promoter regions of genes regulated by ArcA in response to aerobiosis or H2O2 exposure under aerobic conditions (Additional file 2: Table S2) were predicted using the Matrix-scan software [38] available at http://rsat.ulb.ac.be/. The parameters used for the analysis were those given by default by the software. Binding sites with a p-value of ≤ 0.0001 were considered significant and reported as predicted ArcA binding sites.

Biochemical determinations

Overnight cultures of strains 14028s wild type and ΔarcA were diluted (1:100) and cells were grown to OD600 ~ 0.4. At this point, H2O2 (1.0 mM) was added and cells were grown for 20 min. Control cells received no treatment. Experiments were performed in triplicate on different days. After treatment, 6 ml of cultures were withdrawn for each analysis and used for measurement of NADH, glutathione (GSH) and glutathione reductase (GR) activity. NADH was measured using commercially available kits by Abcam. The ratio between reduced glutathione and oxidized glutathione (GSH/GSSG) and GR activity were measured using commercially available kits by Cayman Chemicals. In all cases, measurements were performed following the instructions provided by the manufacturers without modifications.

Measurement of intracellular ROS was performed using the oxidant-sensitive probe H2DCFDA, as previously described, with minor modifications [39]. Briefly, aerobically grown cells in LB at OD600 ~ 0.4 were incubated with 10 µM H2DCFDA. At 10 min intervals aliquots were taken, washed with 10 mM potassium phosphate buffer, pH 7.0, resuspended in the same buffer, and disrupted by sonication. Cell extracts (100 µl) were mixed with 1 ml phosphate buffer and fluorescence was measured using a TECAN Infinite 200 PRO Nanoquant microplate reader (excitation, 480 nm; emission, 520 nm). Emission values were normalized based on the bacterial concentration as determined by the Optical Density (OD) of the culture at 600 nm.

Results

To analyze the role of ArcA in the transcriptional response to aerobiosis and H2O2, the ArcA regulon of S. Typhimurium was determined by microarray analysis. Expression profiles were measured from three independent samples of aerobically grown wild-type (14028s) and ΔarcA strains with or without 1 mM H2O2. After normalization, 3949 genes showed intensity values over the background in at least one array and were included in the analysis. The results were validated by randomly selecting eight genes and measuring the transcript levels by qRT-PCR (Additional file 1: Table S1). A statistically significant correlation was observed between microarray and qRT-PCR data (r2 = 0.7, p-value ≤ 0.0001), despite quantitative differences in the level of change, suggesting that the results obtained by microarray analysis reflect the actual changes in gene expression.

In the wild type strain, expression of 381 and 667 genes was up- or downregulated, respectively, in response to H2O2 under aerobic conditions (fold change ≥ 2, FDR q-value treatment ≤ 0.05, Additional file 2: Table S2). Several genes known to be upregulated by H2O2 and required for its resistance were among the upregulated genes in the wild type and ΔarcA strains, including katE, katG and mntH [40]. A previous study investigated the effect of H2O2 on gene expression in a different S. Typhimurium strain, 4/74 [41]. That study found 309 genes to be upregulated after H2O2 exposure, and 428 genes to be downregulated. The concurrence with our results in strain 14028s is about 30%: 119/381 upregulated 14028s genes and 191/667 downregulated 14028s genes had been found to be similarly regulated in strain 4/74. The observed differences in the number of genes differentially expressed might be explained by several factors including OD600 of treatment (0.4 vs 0.1), time of H2O2 challenge (20 min vs 12 min), threshold for considering a gene differentially expressed (fold change ≥ 2 vs ≥ 3), and different S. Typhimurium strains used in the studies (14028s vs 4/74).

Role of ArcA during aerobic conditions

The expression of 220 and 122 genes was up- or downregulated, respectively, by ArcA under aerobic conditions (Additional file 2: Table S2). Comparison with the ArcA regulon of strain 14028s in anaerobiosis [8] showed that 63 genes were regulated under both conditions, but the expression of 38 genes was regulated in the opposite direction. Of the 220 genes upregulated by ArcA in aerobiosis, only 15 are positively regulated by ArcA under anaerobic conditions (Figure 1A), while of the 122 genes downregulated by ArcA under aerobic conditions, only 10 are also downregulated by ArcA in anaerobiosis (Figure 1B).

To deduce the biological pathways altered in the arcA mutant as compared to the aerobically grown wild type strain under aerobic conditions, a Gene Set Enrichment Analysis (GSEA) was performed using the KEGG database for S. Typhimurium LT2 as a reference. It should
be noted that to determine the pathways regulated by ArcA under aerobic conditions, we compared the transcript levels of the \( \Delta \text{arcA} \) mutant to those in the wild type strain (log\(_2\) \( \Delta \text{arcA} \) aerobic – log\(_2\) wild type aerobic). Thus, pathways repressed by ArcA possess positive NES values (i.e., > 0, glycolysis), while pathways activated by ArcA have negative NES values (i.e., < 0, ABC transporters). GSEA showed that 12 and 8 pathways were up- or downregulated, respectively, by ArcA under aerobic conditions (Table 1). Among the pathways upregulated by ArcA were those implicated in the transport of amino acids and sugars, oligopeptides and metals, including PTS and ABC transporters, among others (Table 1, negative NES values). Under aerobic conditions, ArcA mainly repressed pathways implicated in central metabolism and nucleotide biosynthesis (Table 1, positive NES values). In particular, the transcript levels of genes encoding proteins of the payoff phase of glycolysis [42] and glycerolipid metabolism were higher in the \( \Delta \text{arcA} \) mutant grown under aerobic conditions than in strain 14028s (Figure 2, Addition file 2 Table S2), including \( \text{pykF} \) (pyruvate kinase), \( \text{aceEF-lpdA} \) (pyruvate dehydrogenase complex), \( \text{eno} \) (enolase), \( \text{glpD} \) and \( \text{glpABC} \) (glycerol 3-phosphate dehydrogenase). The data suggests that the aerobically grown \( \Delta \text{arcA} \) mutant presents a higher flux through glycolysis and increased levels of NADH than the wild type strain. Interestingly, genes of the Krebs cycle, which are negatively regulated by ArcA under anaerobic conditions [8], were not repressed in aerobiosis (Table S2). Together, these results indicate that ArcA has a major role in regulating gene expression under aerobic conditions and that the genes regulated in aerobiosis are different from those regulated in anaerobiosis.

### Role of ArcA in the response to H\(_2\)O\(_2\) under aerobic conditions

It has been well established that an aerobically grown \( \Delta \text{arcA} \) strain is sensitive to H\(_2\)O\(_2\) treatment [7-10], however, the genes regulated by ArcA under this condition have not been determined. Our analysis shows that ArcA directly or indirectly regulates the expression of 292 genes in response to H\(_2\)O\(_2\) in aerobically grown cells, 117 and 175 genes were up- or downregulated, respectively (Figure 1, Additional file 2: Table S2). Surprisingly, almost no correlation was observed between the genes regulated by ArcA in aerobiosis with or without H\(_2\)O\(_2\): only 6 genes were upregulated under both conditions (Figure 1A), while 7 genes were downregulated (Figure 1B). Furthermore, comparison of the ArcA regulon in aerobiosis, anaerobiosis [8] and aerobiosis with H\(_2\)O\(_2\) showed that no genes were up or downregulated in all three conditions (Figure 1A and B). This suggests that the genes regulated by ArcA in response to various stimuli are different and do hardly overlap (Figure 1), which underscores the importance and versatility of ArcA-mediated regulation.

To determine the pathways regulated by ArcA in response to H\(_2\)O\(_2\) under aerobic conditions, the intensity values were treated as follows: (log\(_2\) 14028s wild type...
Therefore, in contrast to the pathways regulated by ArcA under aerobic conditions, the pathways positively regulated by ArcA in response to H2O2 under aerobic conditions have positive NES values, while the pathways negatively regulated by ArcA in response to H2O2 under aerobic conditions have negative NES values. Based on the transcriptomic data (Additional file 2: Table S2), GSEA deduced that 10 and 2 pathways were up- or down-regulated, respectively, by ArcA in aerobically grown cells after H2O2 exposure (Table 1). The pathways deduced to be upregulated by ArcA in response to H2O2 are implicated in nucleotide and siderophore biosynthesis, central and glutathione metabolism, among others, while the pathways downregulated by ArcA were PTS and ABC transporters (Figure 2, Table 1). Interestingly, only one gene (ahpF) required for H2O2 degradation was upregulated by ArcA in aerobiosis after H2O2 treatment (Additional file 2: Table S2), suggesting that ArcA is not required for ROS scavenging. Of particular interest are the genes most upregulated by ArcA in aerobiosis with H2O2 in the pathways of nucleotide and glutathione metabolism, coding for the alternative ribonucleotide reductase (nrdEF) and glutathione reductase (gor). In addition, the gene coding for thioredoxin reductase (trxB), required for reduction of oxidized thioredoxin (Trx-[S]2), was also upregulated by ArcA after peroxide.

### Table 1 Pathways differentially regulated (q ≤ 0.25) by ArcA in response to H2O2 and aerobiosis as determined by GSEA

| Gene set                              | Size of gene set^a| Genes regulated FDR q-value | NES^H | NES^G |
|---------------------------------------|-------------------|-----------------------------|-------|-------|
| Glycerophospholipid metabolism        | 25                | 5                           | 5     | 0.001 | 2.16  |
| Purine metabolism                     | 73                | 19                          | 35    | 0.003 | 2.06  |
| Pyrimidine metabolism                 | 50                | 14                          | 18    | 0.044 | 1.79  |
| Lipopolisaccharide biosynthesis       | 26                | 17                          |       | 0.004 | 1.95  |
| Glutathione metabolism                | 16                | 8                           |       | 0.005 | 1.91  |
| Bacterial invasion of epithelial cells | 8                 | 8                           | 5     | 0.002 | −1.9   |
| Glycolysis/glucoseogenesis            | 33                | 10                          | 10    | 0.250 | 0.46  |
| Biosynthesis of siderophore group non-ribosomal peptides | 5 | 4 | 0.067 | 1.69 |
| Glycerolipid metabolism               | 10                | 1                           | 1     | 0.083 | 1.68  |
| Flagellar assembly                    | 34                | 7                           |       | 0.189 | 1.5   |
| Phosphotransferase system (PTS)       | 32                | 17                          | 17    | 0.001 | −1.88 |
| ABC transporters                      | 124               | 46                          | 29    | 0.005 | −1.83 |
| Salmonella infection                  | 16                | 8                           |       | 0.001 | −2.04 |
| Bacterial secretion system            | 28                | 12                          |       | 0.005 | −1.79 |
| Bacterial chemotaxis                  | 22                | 6                           |       | 0.049 | −1.63 |
| Two-component system                  | 88                | 11                          |       | 0.096 | −1.56 |
| Cyanocobalamin acid metabolism        | 6                 | 3                           |       | 0.129 | −1.52 |
| Ascorbate and aldarate metabolism     | 8                 | 3                           |       | 0.117 | −1.52 |
| Amino sugar and nucleotide sugar metabolism | 50           | 14                          |       | 0.213 | −1.43 |
| Fructose and mannose metabolism       | 34                | 14                          |       | 0.233 | −1.43 |
| Starch and sucrose metabolism         | 26                | 14                          |       | 0.244 | −1.39 |
| Ribosome                              | 45                | 28                          |       | 0.004 | 2.01  |
| Riboflavin metabolism                 | 8                 | 3                           |       | 0.113 | 1.63  |
| Cysteine and methionine metabolism    | 24                | 8                           |       | 0.137 | 1.59  |

^aBased on the KEGG database for *S. Typhimurium* LT2.

^bNumber of genes predicted to contribute to the enrichment in aerobic growth in rich media.

^cNumber of genes predicted to contribute to the enrichment in response to H2O2.

^d-q-value for the pathway predicted to be regulated by ArcA in aerobic growth in rich media.

^e-q-value for the pathway predicted to be regulated by ArcA in response to H2O2.

^fNormalized enrichment score for pathways under aerobic conditions. Positive values indicate pathways negatively regulated by ArcA, while negative values indicate pathways that are up-regulated by ArcA.

^gNormalized enrichment score for pathways under aerobic conditions after H2O2 exposure. Positive values indicate pathways positively regulated by ArcA, while negative ones indicate pathways that are downregulated by ArcA.

^hNormalized enrichment score.
exposure. In *E. coli*, *nrdEF* is upregulated in response to H$_2$O$_2$ [43,44] and allows replication in iron-limiting conditions when manganese is present [44]. Gor and TrxB are required for the turnover of oxidized glutathione (GSSG) and Trx-[S]$_2$, respectively (reduction from GSSG to reduced glutathione (GSH), and Trx-[S]$_2$ to reduced thioredoxin (Trx-[SH]$_2$), respectively), which participate in the reduction of disulfide bonds [45]. This suggests that in aerobically grown cells exposed to H$_2$O$_2$, ArcA regulates the GSH/GSSG and Trx-[S]$_2$/Trx-[SH]$_2$ ratio, modulating the redox status of the cell, and the expression of *nrdEF*.

**Figure 2** Effect of ArcA on the expression of genes of central metabolism, sugar uptake and reductive pathways in aerobiosis with or without H$_2$O$_2$. Selected genes involved in glycolysis, glycerolipid-, GSH- and thioredoxin- (Trx-[SH]$_2$) metabolism, ABC transport and PTS are depicted. Statistically significant differences between strains 14028s and ΔarcA in aerobiosis with or without H$_2$O$_2$ are shown (Table S2). Fold changes are depicted for the aerobically grown wild type strain after H$_2$O$_2$ treatment (upper value), ΔarcA strain after H$_2$O$_2$ treatment (middle value), and aerobically grown untreated ΔarcA/14028s (lower value). Boxes indicate genes regulated by ArcA (Fold change ≥ 2, FDR q-value ≤ 0.05) in aerobiosis (blue), aerobiosis with H$_2$O$_2$ (red) and under both conditions (black).
Promoter analysis
To determine the genes that may be directly regulated by ArcA, we identified the subset of ArcA-dependently differentially expressed genes in aerobiosis with or without H$_2$O$_2$ with predicted ArcA-binding sites in the upstream regions (Additional file 2: Table S2), using the sequenced genome of S. Typhimurium 14028s [37] and Matrix-scan [38], as detailed in Methods. The analysis predicted that ArcA directly regulates the expression of 6 genes in aerobiosis with H$_2$O$_2$ and 19 genes in aerobically grown cells (Table 2).

Biochemical analysis of the ΔarcA strain
The microarray analysis predicted that in response to H$_2$O$_2$ under aerobic conditions, ArcA regulates the expression of genes implicated in GSH metabolism. It also predicted that in aerobiosis, ArcA regulates expression of genes coding proteins involved in glycolysis (Table 1 and Additional file 2: Table S2). To evaluate if the changes in gene expression correlated with changes in the products of these pathways, we determined the levels of GSH, glutathione reductase (GR) activity, NADH and the products of these pathways, we determined the levels of GSH, glutathione reductase (GR) activity, NADH and total intracellular ROS in the wild type and ΔarcA strains in aerobiosis with and without H$_2$O$_2$.

The gene gor was upregulated by ArcA under aerobic conditions with H$_2$O$_2$ (Figure 2, Additional file 2: Table S2), therefore decreased levels of both GSH and GR activity in the arcA mutant exposed to H$_2$O$_2$ under aerobic conditions were expected. The levels of GR activity were indeed lower in the aerobically grown ΔarcA strain after H$_2$O$_2$ treatment, although the levels of GR activity were also decreased in the ΔarcA mutant under aerobic conditions (Figure 3A). GSH remained almost unaltered in the wild type strain after treatment with the toxic compound, conversely, the aerobically grown ΔarcA mutant treated with H$_2$O$_2$ showed significantly reduced levels of GSH and increased GSGG, consistent with lower GR activity (Figure 3A and B). In agreement, the GSH/GSSG ratio was lower in the aerobically grown ΔarcA strain after H$_2$O$_2$ treatment (Figure 3C), while the levels of total glutathione were similar between the wild type and ΔarcA mutant strains (4.68 and 4.45 μmol/mg. protein, respectively). This indicates that GSH turnover is altered in an aerobically grown ΔarcA strain with H$_2$O$_2$ due to lower GR activity.

The transcript levels of genes coding the pyruvate dehydrogenase complex (PDH), proteins of the payoff phase of glycolysis [42] and sugar uptake were higher in the aerobically grown ΔarcA mutant than in the wild type strain (Figure 2, Additional file 2: Table S2). This suggests that under aerobic conditions a ΔarcA strain has a higher flux through glycolysis, which in turn could result in higher levels of acetyl-CoA and an elevated NADH generation in the Krebs cycle. As predicted, the NADH/NAD$^+$ ratio was significantly lowered in the aerobically grown wild type strain after peroxide treatment (Figure 3D), compared to untreated aerobically grown wild type cells. In the ΔarcA mutant, the NADH/NAD$^+$ ratio was higher than in the wild type strain in aerobically grown cells before and after H$_2$O$_2$ treatment (Figure 3D). Although there was an overall decrease in the NADH/ NAD$^+$ ratio in the ΔarcA strain after H$_2$O$_2$ treatment, the ratio remained 2-fold higher than in wild type cells under aerobic conditions without H$_2$O$_2$ treatment.

In E. coli, one of the sources of O$_2^-$ is oxidation of the respiratory electron transport chain and the conversion of NADH to NAD$^+$ [46]. Since under aerobic growth conditions a ΔarcA strain has higher levels of NADH (Figure 3D) and ndh transcript than the wild type strain (Additional file 2: Table S2), we hypothesized that a ΔarcA mutant might present increased levels of total ROS. In agreement, in an aerobically grown ΔarcA strain, total ROS was increased as compared to the isogenic wild type strain under the same condition (Figure 3E), indicating that the absence of ArcA generates a metabolic imbalance which leads to increased levels of ROS.

In order to complement the ΔarcA mutation, we first evaluated the mechanism by which ArcA regulates gene expression in response to ROS. Our results show that in S. Typhimurium 14028s, arcA expression is not increased either with H$_2$O$_2$ or hypochlorous acid (Additional file 1: Figure S1A). In addition, the levels of ArcA also remained constant after exposure to both ROS (Additional file 1: Figure S1B). This suggests that rather than changes in expression, ArcA is activated in response to ROS, most likely by phosphorylation of residue D54. To test this hypothesis, the ΔarcA mutant strain was complemented in trans with the wild type gene and a version coding a substitution of residue D54 of ArcA (D54A), and the number of colony forming units (CFU/ml) was determined after H$_2$O$_2$ exposure. As predicted, only complementation with the wild type gene resulted in similar CFU/ml as in strain 14028s (Additional file 1: Figure S1C, however, there were also differences in the number of CFU/ml at the initial time points. This is most likely caused by increased levels of ArcA due to complementation with a high copy number vector. Since the effect of ArcA is pleiotropic and its levels remain constant throughout all stresses evaluated (Additional file 1: Figure S1 A and B), achieving wild type levels of ArcA is required to properly address its role in the response to ROS.

Discussion
Several reports have demonstrated that the global regulator ArcA is required for H$_2$O$_2$ resistance [7-10], however, only a few have evaluated its role on regulating gene expression under this condition [27,28]. One study conducted in E. coli used a proteomic approach to
| Gene ID     | Gene ID     | Gene name | Fold change H2O2/control | Fold change control | Strand | Position | Sequence          | p-value | Function                        |
|------------|------------|-----------|--------------------------|---------------------|--------|----------|-------------------|---------|---------------------------------|
| STM0958    | STM14_1080 | trxB      | 4.93                     | 1.9                 | 1.35   | -        | GTTAAACAATATGTGT   | 1.00E-05| thioredoxin reductase            |
| STM1520    | STM14_1838 | marR      | 1.75                     | -1.38               | -1.02  | -        | GTCACTAAATGAAT     | 9.50E-05| DNA-binding transcriptional repressor MarR |
| STM1586    | STM14_1918 |           | 5.55                     | 2.75                | 1.06   | +        | GTTAAAGAATGTC      | 9.50E-05| putative periplasmic protein     |
| STM3216    | STM14_3893 | tsr       | 1.17                     | -1.65               | -1.31  | -        | GTTAACTTTCTTA      | 8.10E-06| putative methyl-accepting chemotaxis protein |
| STM2445    | STM14_3003 | ucpA      | -5.34                    | -1.83               | -1.86  | +        | GTTAAATGGAGTGAAT   | 1.20E-05| short chain dehydrogenase        |
| STM1795    | STM14_2170 | gluD      | -6.28                    | -1.34               | -1.94  | -        | GTTAACTCCCGCT      | 9.50E-05| putative glutamic dehydrogenase-like protein |
| STM4087    | STM14_4913 | gplF      | -1.63                    | -5.7                | 8.77   | +        | GTTAAATGAAATTGATT  | 1.00E-05| glycerol diffusion              |
| STM1771    | STM14_2141 | chaA      | -1.77                    | -5.97               | 3.31   | -        | GTTAAATTITGGAA     | 8.00E-05| calcium/sodium/proton antipporter |
| STM1125    | STM14_1281 | putP      | -8.81                    | -3.4                | -3.46  | +        | GTTAAACCTTTAAA     | 9.50E-05| major sodium/proline symporter   |
| STM1091    | STM14_1237 | sopB      | -2.17                    | -3.57               | -5.89  | +        | GTTAAACCCTTGAA     | 8.00E-05| secreted effector protein        |
| STM2086    | STM14_3463 | sprB      | -6.06                    | -2.32               | -3.51  | +        | GTTAAATGAAAGGAGA   | 8.10E-06| transcriptional regulator        |
| STM4405    | STM14_5290 | ytlJ      | -3.2                     | -1.45               | -2.31  | -        | GTTAACTATATGTC     | 3.30E-05| putative transcriptional regulator |
| STM4535    | STM14_5449 |           | -2.7                     | -1.04               | -2.76  | -        | GTTAAACAGAGGAGA    | 9.50E-05| putative PTS permease            |
| STM4467    | STM14_5361 |          | -1.83                    | 1.02                | -2.07  | -        | GTTAAATTITGGTT     | 6.50E-06| arginine deiminase              |
| STM1130    | STM14_1293 | nanM      | -2.58                    | 1.01                | -2.92  | +        | GATACTCCATGTA      | 8.00E-05| putative inner membrane protein  |
| STM4165    | STM14_5006 | rsld      | 2.75                     | 1.69                | 2.74   | -        | GTTAAACACATGCCA    | 1.20E-05| anti-RNA polymerase sigma 70 factor |
| STM1728    | STM14_2091 | ycgG      | 1.68                     | 1.95                | -2.11  | -        | GTTAAATGCGGTGT     | 1.50E-05| putative cytoplasmic protein     |
| STM0292    | STM14_0341 |           | 1.18                     | 2.12                | 2.18   | -        | GTTACATCAATGAG     | 6.80E-05| putative RRS-like protein        |
| STM2220    | STM14_2744 | yegG      | 5.84                     | 2.41                | 2.94   | +        | GTCAATGATGATTGTA   | 6.80E-05| hypothetical protein            |
| STM1770    | STM14_2140 | chaB      | 2.07                     | 3.64                | -2.12  | +        | GTTAAATTTITGGAGA   | 8.00E-05| cation transport regulator       |
| STM1211    | STM14_1385 | ndh       | 7.09                     | 3.58                | 2.24   | -        | GTTAAATTAAAGTGA    | 1.10E-06| respiratory NADH dehydrogenase 2 |
| STM1746    | STM14_2110 | oppA      | -7.87                    | -2.46               | -3.08  | +        | GTTAAACAAATGTA     | 1.00E-05| oligopeptide transport protein   |
| STM1818    | STM14_2199 | fadD      | -1.56                    | -1.85               | 2.56   | +        | GTTAAATAATAGTGA    | 1.00E-05| long-chain-fatty-acid-CoA ligase |
| STM3692    | STM14_4451 | lidP      | -13.27                   | -2.11               | -6.83  | -        | GTTAAACCAGATGTA    | 2.00E-06| L-lactate permease              |
| STM1303    | STM14_1582 | argD      | -2.63                    | -1.11               | -2.95  | -        | GTTATAATTTGTTGTA   | 5.20E-06| bifunctional succinylornithine transaminase |

*a* Location of the binding sequence is in bp upstream of the translation start site in the genome of S. Typhimurium 14028s [37].

*b* p-value estimates the significance of the weight associated to each site [38].
evaluate the mechanism underlying the role of ArcA in response to ROS [7]. Herein, we report the first genome-wide study addressing the role of ArcA in response to H2O2 under aerobic conditions. ArcA regulates different genes after ROS exposure in aerobiosis, under aerobic growth in rich media and under anaerobiosis (Figure 1, 2 and 4; Additional file 2: Table S2). In this discussion, we will focus on the genes and pathways regulated by ArcA that contribute to ROS resistance of S. Typhimurium. A full list of the genes regulated by ArcA in aerobiosis with and without H2O2 is provided in Additional file 2: Table S2.

Role of ArcA in ROS scavenging

The S. Typhimurium genome codes for several genes that degrade H2O2 or organic hydroperoxide, including catalases (katG, katE and katN), alkyl hydroperoxide reductases (ahpCF and tsaA) [47] and a glutathione-dependent peroxidase (btuE) [48]. Only ahpF and katE are predicted to be regulated by ArcA in aerobiosis with or without H2O2, respectively (Additional file 2: Table S2).

Expression of ahpF and katE is also known to be regulated by RpoS [49,50]. Neither katN nor sodA, previously described as members of the ArcA regulon under anaerobic conditions [8,51], were found to be regulated by ArcA under aerobic conditions with or without H2O2 treatment. These results are in agreement with studies in E. coli that demonstrate that an arcA mutant does not show defects in H2O2 scavenging [7].

Role of ArcA in maintaining GSH and thioredoxin levels

ArcA positively regulates the expression of the genes gor (GR) and trxB (thioredoxin reductase) in aerobiosis with H2O2, but not without the toxic compound (Figure 2 and 4 Additional file 2: Table S2). However, the levels of GR activity were lower in the arcA mutant strain grown under aerobic conditions (Figure 3A), but not the levels of GSH turnover (Figure 3B). This suggests that there are other unidentified factors that alter GR activity in the arcA mutant grown under aerobic conditions, since there are no differences in the transcript levels of the gene gor between strains 14028s and ΔarcA (Additional
file 2: Table S2). In addition, this indicates that the lower levels of GR activity in the arcA mutant grown under aerobic conditions are sufficient to cope with GSH turnover, and the effect is only evident when GSH oxidation is increased, as when cells are exposed to peroxide.

In E. coli, OxyR regulates gor expression [6], while the expression of trxB has not been determined under this condition. Two putative ArcA binding sites were predicted at the promoter region of trxB (Table 2). Glutathione and thioredoxin reductases are required to reduce GSSG and thioredoxin in a NADPH-dependant manner, which in their reduced form participate in the reduction of cellular disulfide bonds [45] and of oxidized glutaredoxin. An E. coli Δgor mutant has a slight sensitivity towards paraquat and cumene hydroperoxide [52], while in stationary phase a ΔtrxB strain shows H2O2 sensitivity [53]. However, a double Δgor ΔtrxB mutant grows extremely poorly under aerobic conditions and presents increased alkaline phosphatase activity, indicative of increased disulfide bond formation, most likely due to increased ROS [54]. Since an aerobically grown ΔarcA mutant treated with H2O2 has lower transcript levels of gor and trxB, lower GR activity and lower GSH levels (Figure 2 and 3A, B and C), this might result in increased disulfide bond formation, protein inactivation and contribute to the increased sensitivity towards ROS. Since GSH is abundant in the cell and is readily oxidized by H2O2, this leads to a decrease in the levels of reduced glutathione, shifting the target of oxidation from GSH to essential macromolecules, leading to cell death [55]. This may occur earlier in a ΔarcA mutant, as its level of “protective” GSH is low, caused by decreased GR activity.

**ArcA and nucleotide metabolism**

The pathways that showed the highest changes in the ΔarcA mutant treated with H2O2 under aerobic conditions were purine and pyrimidine metabolism (Figure 4, Table 1). The major differences are found in the expression of nrdAB and nrdEFHI operons, coding for aerobic and alternative aerobic ribonucleotide reductase, respectively. In E. coli and S. Typhimurium, NrdAB is indispensable for growth under aerobic conditions while NrdEF is not functional [56]. In the aerobically grown wild type strain, nrdAB was repressed while nrdEF was up-regulated after H2O2 exposure, in agreement with studies in E. coli and S. Typhimurium 4/74 [41,43], while the regulation was lost in the ΔarcA mutant under the same conditions (Additional file 2: Table S2). NrdEF is usually repressed by Fur. However, in response to H2O2, this Fur repression is abolished and the apoprotein form of IscR upregulates expression of the operon [44]. This suggests that in response to H2O2 under aerobic conditions, ArcA may act together with Apo-IscR, up-regulating the nrdEFHI operon.
ArcA and carbon metabolism

Under aerobic conditions, the transcript levels of genes coding proteins of glycerolipid metabolism, glycolysis and the PDH complex were higher in the ΔarcA mutant than in the wild type strain (Figure 2 and 4, Additional file 2: Table S2). This suggests that the flux through glycolysis and the levels of acetyl-CoA could be increased in the ΔarcA strain. Two studies conducted in *E. coli* measured the flux through the PDH complex in a ΔarcA mutant under aerobic conditions with different results. One showed that there was an increase in the flux through the TCA cycle [14] while in the other no differences were observed [57], although both studies determined that there was an increase in the flux through the TCA cycle in aerobic conditions (Figure 3D). Since NADH can reduce Fe³⁺ to Fe²⁺ in vitro [56], and elevated NADH levels result in increased sensitivity towards H₂O₂ [58], the higher basal levels of NADH in the ΔarcA mutant in aerobic conditions and after H₂O₂ treatment may increase Fe²⁺ turnover, fueling the Fenton reaction (the formation of OH−, and Fe³⁺ from the nonenzymatic reaction of Fe²⁺ with H₂O₂) and leading to higher levels of ROS-derived damage.

In the respiratory chain, NADH dehydrogenase II (encoded by *ndh*) generates O₂− and H₂O₂ by oxidation of its reduced FADH₂ cofactor [58]. In an aerobically grown ΔarcA strain, the levels of NADH and the *ndh* transcript (Additional file 2: Table S2) are higher than in the wild type strain under the same condition (Figure 3D). We therefore speculated that production of intracellular ROS might be increased. In agreement, a ΔarcA mutant presents statistically significant increased levels of total ROS as compared to the wild type strain 14028s (Figure 3E). These higher levels of ROS might present further disadvantages for the bacterium when exposed to H₂O₂. However, several other sources of intracellular ROS besides NADH dehydrogenase II may also contribute to the higher levels of ROS observed in the ΔarcA mutant, such as fumarate-reducing flavoenzymes [59].

Conclusion

We identified the ArcA regulon in *S. Typhimurium* under aerobic growth with and without H₂O₂, and show that ArcA coordinates a response that includes changes in cellular-, glutathione-, thioredoxin-, NADH- and glycerolipid metabolism. These changes contribute to H₂O₂ resistance by modulating the reducing potential of the cell.

Additional files

**Additional file 1: Probing the ArcA regulon under aerobic/ROS conditions in *Salmonella enterica* serovar Typhimurium.**

A) Supplementary methods. B) Figure S1: Characterization of the mechanism of ArcA in response to ROS. Measurement of the transcript and protein levels of *arcA* by qRT-PCR and Western blot, respectively. Determination of CFU/ml in strains 14028s, ΔarcA, ΔarcA-cat/pBRarcA, and ΔarcA-cat/pBRarcAΔcat, after H₂O₂ exposure. C) Table S1: Validation of microarray data using qRT-PCR of randomly selected genes. Fold changes are given for the selected genes in response to hydrogen peroxide in the different genetic backgrounds as determined by qRT-PCR and microarray analysis. D) Supplementary references [60].

**Additional file 2: Table S2.** Table of genes that showed intensity values over the background. Fold changes are given for every gene in response to H₂O₂ in the different genetic backgrounds.

Competing interests

The author(s) declare that they have no competing interests.

Author’s contributions

EHM and CPS conceived the project. EHM and PD conducted the analysis of microarray data and prediction of regulated pathways; EHM, BC and ILC performed the experiments. FG, RL and SP conducted partial data analysis. EHM, SP, MM and CPS wrote the paper. All authors read and approved the final manuscript. The authors declare no conflict of interest.

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Author details

1. Laboratorio de Microbiología Molecular, Facultad Ciencias Biológicas, Universidad Andres Bello, Santiago, Chile. 2. Department of Microbiology and Molecular Genetics, B240 Medical Sciences Building, University of California, Irvine, CA 92697, USA. 3. Present address: Great Lakes Bioenergy Research Center, University of Wisconsin-Madison, Madison, Wisconsin, USA. 4. Present address: Department of Biomolecular Chemistry, University of Wisconsin-Madison, Madison, Wisconsin, USA.

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