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

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Abstract

Background: Hydrogen peroxide (H_2O_2) 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 H_2O_2 by querying gene expression and other physiological changes in wild type and $\Delta arcA$ strains.

Results: In the $\Delta arcA$ strain, expression of 292 genes showed direct or indirect regulation by ArcA in response to H_2O_2, of which 141were also regulated in aerobiosis, but in the opposite direction. Gene set enrichment analysis (GSEA) of the expression data from WT and $\Delta arcA$ strains, revealed that, in response to H_2O_2 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, H_2O_2 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 (O_2^-) by phagocytic NADPH

oxidase. O_2^- 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 O_2^- react to generate H_2O_2 [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 H_2O_2 and molecular oxygen from O_2^- [3-5].

The response of the bacterium to H_2O_2 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

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H_2O_2 resistance [7-10]. Furthermore, in *E. coli* H_2O_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 tretamer 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_2O_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_2O_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_2O_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_2O_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_2O_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_2O_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_2O_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 N_6 (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 $\log_2$ values. For each array, the background was calculated as follows: $\log_2$ median intensity value for negative control probes + (3 * $\log_2$ 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 Δ*arcA* ((wild type treated/wild type control)/(Δ*arcA* treated/ Δ*arcA* control)) were considered to be differentially regulated in response to H_2O_2. Genes with a q-value of ≤ 0.05 for genotype and a fold change of ≥ 2 between strains Δ*arcA* and wild type (Δ*arcA* 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 H_2O_2 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 H_2O_2, the $\log_2$ values of all replicas were averaged and treated as follows: ($\log_2$ 14028s wild type H_2O_2 aerobic - $\log_2$ 14028s wild type aerobic) - ($\log_2$ Δ*arcA* H_2O_2 aerobic - $\log_2$ Δ*arcA* aerobic). Positive Normalized Enrichment Score (NES) values represent pathways up-regulated by ArcA, while negative NES values represent pathways negatively regulated upon H_2O_2 treatment under aerobic conditions. To determine the pathways regulated under aerobic conditions, the $\log_2$ values of all replicas of untreated cells were averaged and treated as follows: ($\log_2$ Δ*arcA* aerobic - $\log_2$ 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 M×3000P 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 H_2O. 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 *ompW* promoter region, which was shown to be functional [28]. The upstream sequences of the genes regulated by ArcA in response to aerobiosis or H_2O_2 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 H_2O_2 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 Δ*arcA* were diluted (1:100) and cells were grown to $OD_{600} \sim 0.4$. At this point, H_2O_2 (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 H_2DCFDA, as previously described, with minor modifications [39]. Briefly, aerobically grown cells in LB at $OD_{600} \sim 0.4$ were incubated with 10 μM H_2DCFDA. 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 H_2O_2, the ArcA regulon of
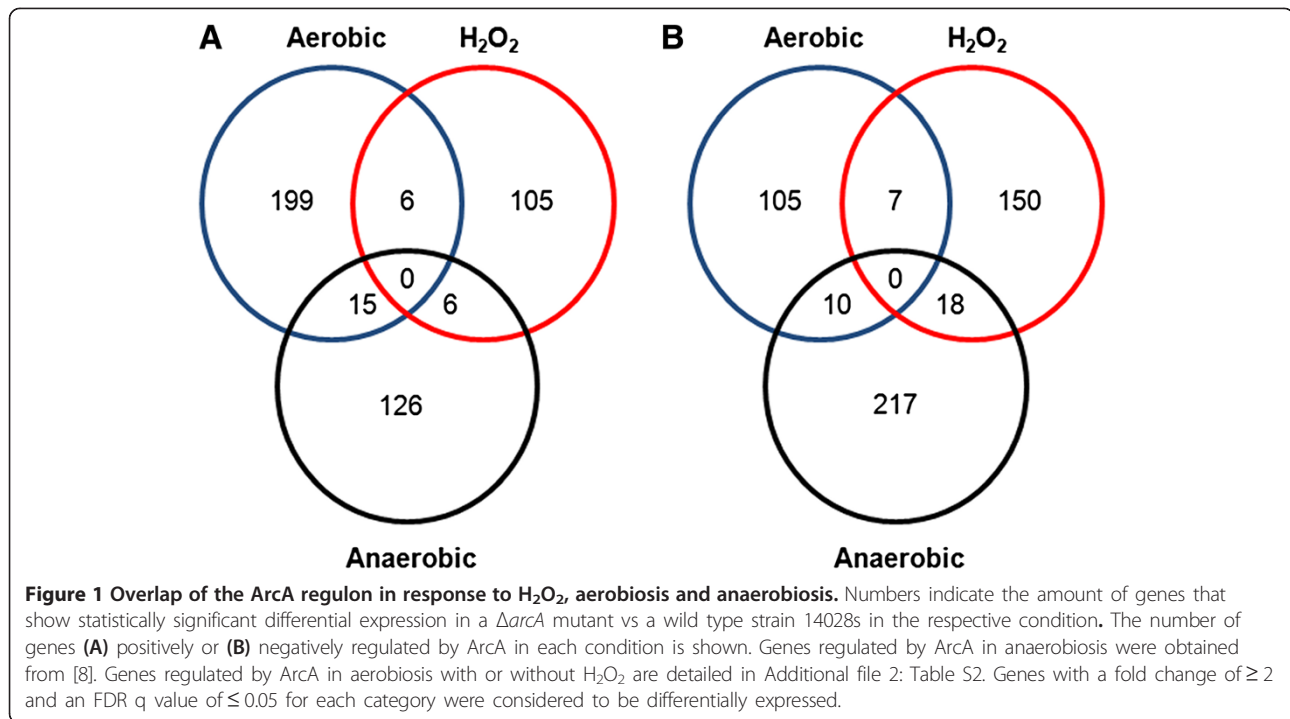
S. Typhimurium was determined by microarray analysis. Expression profiles were measured from three independent samples of aerobically grown wild-type (14028s) and Δ*arcA* strains with or without 1 mM H_2O_2. After normalization, 3949 genes showed intensity values over the background in at least one array 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 ($r^2 = 0.7$, 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 H_2O_2 under aerobic conditions (fold change ≥ 2, FDR q-value treatment ≤ 0.05, Additional file 2: Table S2). Several genes known to be upregulated by H_2O_2 and required for its resistance were among the upregulated genes in the wild type and Δ*arcA* strains, including *katE*, *katG* and *mntH* [40]. A previous study investigated the effect of H_2O_2 on gene expression in a different *S.* Typhimurium strain, 4/74 [41]. That study found 309 genes to be upregulated after H_2O_2 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 OD_{600} of treatment (0.4 vs 0.1), time of H_2O_2 challenge (20 min vs 12 min), threshold for considering a gene differentially expressed (fold change of ≥ 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

Figure 1 Overlap of the ArcA regulon in response to H₂O₂, aerobiosis and anaerobiosis. Numbers indicate the amount of genes that show statistically significant differential expression in a $\Delta arcA$ mutant vs a wild type strain 14028s in the respective condition. The number of genes **(A)** positively or **(B)** negatively regulated by ArcA in each condition is shown. Genes regulated by ArcA in anaerobiosis were obtained from [8]. Genes regulated by ArcA in aerobiosis with or without H₂O₂ are detailed in Additional file 2: Table S2. Genes with a fold change of ≥ 2 and an FDR q value of ≤ 0.05 for each category were considered to be differentially expressed.

be noted that to determine the pathways regulated by ArcA under aerobic conditions, we compared the transcript levels of the *arcA* mutant to those in the wild type strain (log_2 $\Delta arcA$ 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 *arcA* mutant grown under aerobic conditions than in strain 14028s (Figure 2, Addition file 2 Table S2), including *pykF* (pyruvate kinase), *aceEF-lpdA* (pyruvate dehydrogenase complex), *eno* (enolase), *glpD* and *glpABC* (glycerol 3-phosphate dehydrogenase). The data suggests that the aerobically grown $\Delta arcA$ 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 H₂O₂ under aerobic conditions

It has been well established that an aerobically grown $\Delta arcA$ strain is sensitive to H₂O₂ 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 H₂O₂ 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 H₂O₂: 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 H₂O₂ 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 H₂O₂ under aerobic conditions, the intensity values were treated as follows: (log_2 14028s wild type

Table 1 Pathways differentially regulated (q ≤ 0.25) by ArcA in response to H$_2$O$_2$ and aerobiosis as determined by GSEA

Gene set	Size of gene set[A]	Genes regulated		FDR q-value		NES[H]	
		Aerobic[B]	H$_2$O$_2$[C]	Aerobic[D]	H$_2$O$_2$[E]	Aerobic[F]	H$_2$O$_2$[G]
Glycerophospholipid metabolism	25	5	5	0.001	0.000	2.16	2.29
Purine metabolism	73	19	35	0.003	0.000	2.06	2.22
Pyrimidine metabolism	50	14	18	0.044	0.003	1.79	2.02
Lipopolisaccharide biosynthesis	26		17		0.004		1.98
Glutathione metabolism	16		8		0.005		1.94
Bacterial invasion of epithelial cells	8	8	5	0.002	0.012	−1.9	1.87
Glycolysis/gluconeogenesis	33	10	10	0.250	0.022	0.46	1.81
Biosynthesis of siderophore group non-ribosomal peptides	5		4		0.067		1.69
Glycerolipid metabolism	10	1	1	0.083	0.109	1.68	1.6
Flagellar assembly	34		7		0.189		1.5
Phosphotransferase system (PTS)	32	17	17	0.001	0.000	−1.88	−2.26
ABC transporters	124	46	29	0.005	0.022	−1.83	−1.86
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	
Cyanoamino 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	

[A]Based on the KEGG database for *S*. Typhimurium LT2.
[B]Number of genes predicted to contribute to the enrichment in aerobic growth in rich media.
[C]Number of genes predicted to contribute to the enrichment in response to H$_2$O$_2$.
[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 H$_2$O$_2$.
[F]Normalized 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.
[G]Normalized enrichment score for pathways under aerobic conditions after H$_2$O$_2$ exposure. Positive values indicate pathways positively regulated by ArcA, while negative ones indicate pathways that are downregulated by ArcA.
[H]Normalized enrichment score.

H$_2$O$_2$ aerobic - log$_2$ 14028s wild type aerobic) - (log$_2$ $\Delta arcA$ H$_2$O$_2$ aerobic - log$_2$ $\Delta arcA$ aerobic). Therefore, in contrast to the pathways regulated by ArcA under aerobic conditions, the pathways positively regulated by ArcA in response to H$_2$O$_2$ under aerobic conditions have positive NES values, while the pathways negatively regulated by ArcA in response to H$_2$O$_2$ 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 H$_2$O$_2$ exposure (Table 1). The pathways deduced to be upregulated by ArcA in response to H$_2$O$_2$ 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 H$_2$O$_2$ degradation was upregulated by ArcA in aerobiosis after H$_2$O$_2$ 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 H$_2$O$_2$ 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
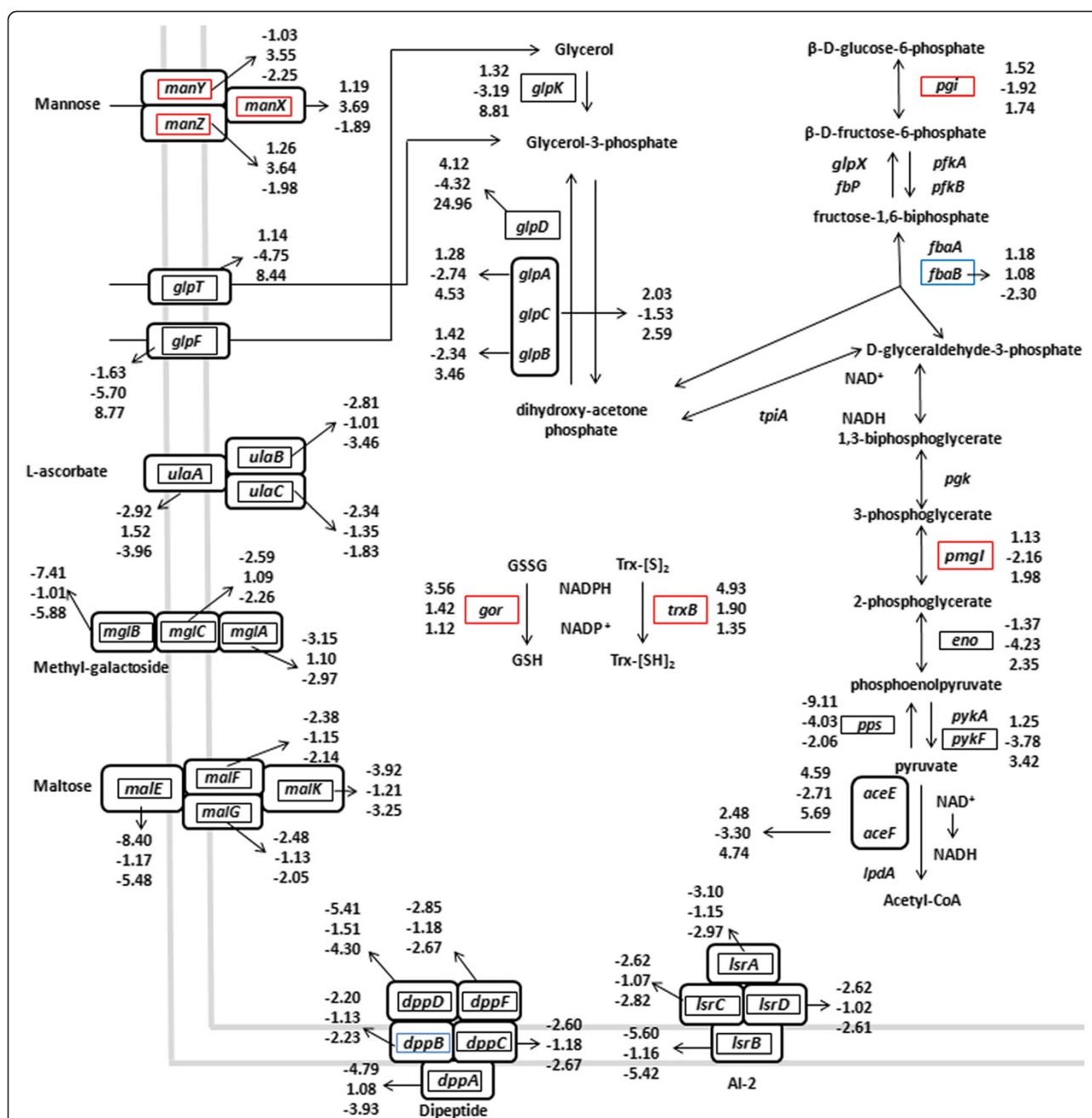
Figure 2 Effect of ArcA on the expression of genes of central metabolism, sugar uptake and reductive pathways in aerobiosis with or without H$_2$O$_2$. Selected genes involved in glycolysis, glycerolipid-, GSH- and thioredoxin- (Trx-[SH]$_2$) metabolism, ABC transport and PTS are depicted. Statistically significant differences between strains 14028s and Δ*arcA* 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), Δ*arcA* strain after H$_2$O$_2$ treatment (middle value), and aerobically grown untreated Δ*arcA*/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).

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*.

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_2O_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_2O_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_2O_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 total intracellular ROS in the wild type and ΔarcA strains in aerobiosis with and without H_2O_2.

The gene *gor* was upregulated by ArcA under aerobic conditions with H_2O_2 (Figure 2, Additional file 2: Table S2), therefore decreased levels of both GSH and GR activity in the *arcA* mutant exposed to H_2O_2 under aerobic conditions were expected. The levels of GR activity were indeed lower in the aerobically grown ΔarcA strain after H_2O_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_2O_2 showed significantly reduced levels of GSH and increased GSSG, 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_2O_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_2O_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_2O_2 treatment (Figure 3D). Although there was an overall decrease in the NADH/NAD$^+$ ratio in the ΔarcA strain after H_2O_2 treatment, the ratio remained 2-fold higher than in wild type cells under aerobic conditions without H_2O_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_2O_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_2O_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.
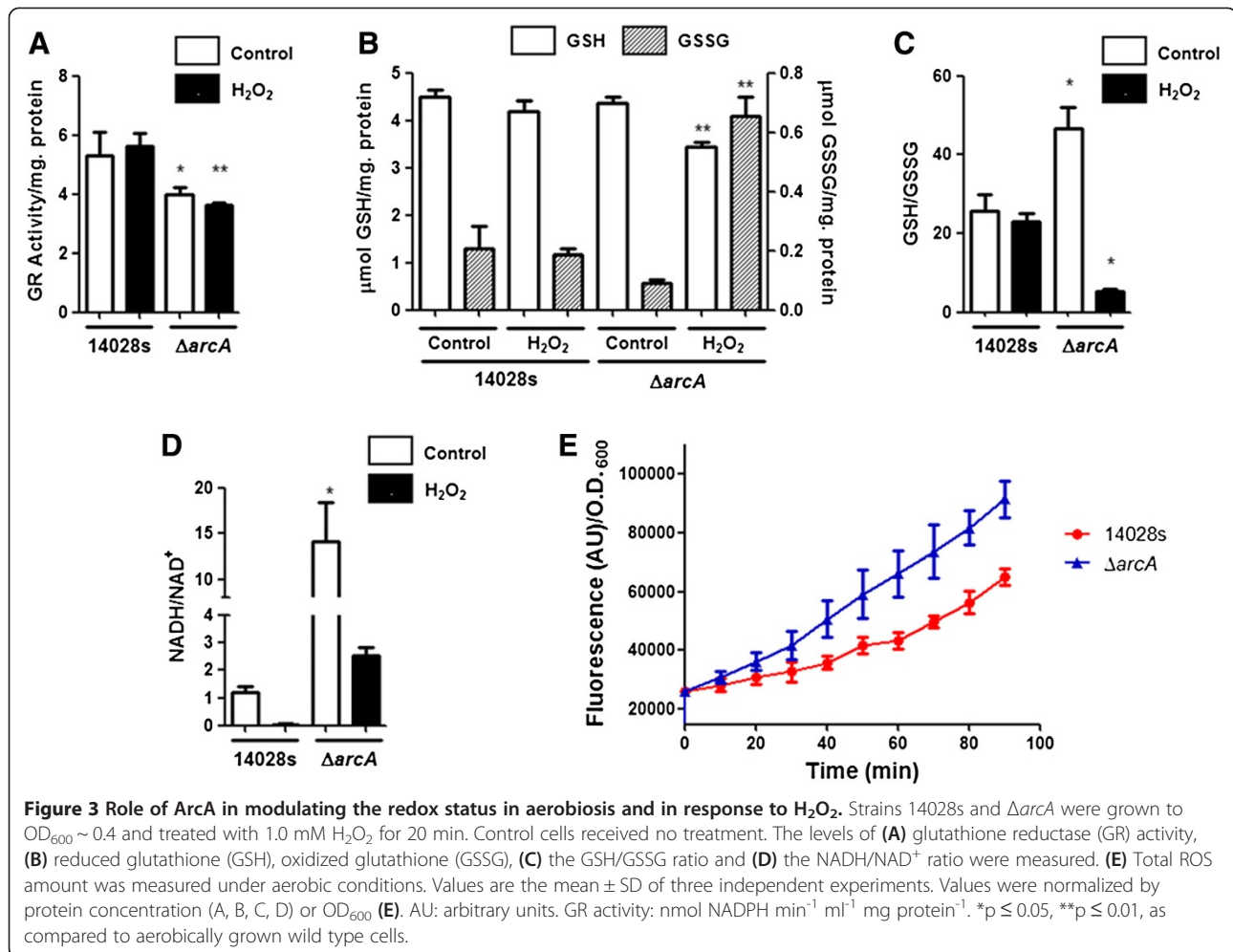
Discussion

Several reports have demonstrated that the global regulator ArcA is required for H_2O_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

Table 2 Genes differentially expressed by ArcA under aerobic conditions with or without H_2O_2 that have predicted ArcA binding sites

Gene ID LT2	Gene ID 14028s	Gene name	Fold change H_2O_2/control 14028s	Fold change H_2O_2/control $\Delta arcA$	Fold change control $\Delta arcA$/14028s	Strand	Position[A]	Sequence 5' - 3'	p-value[B]	Function
STM0958	STM14_1080.J	trxB	4.93	1.9	1.35	-	94	GTTAACAATATGTGT	1.00E-05	thioredoxin reductase
						+	85	GTTAACAAAATCGTT	5.70E-05	
STM1520	STM14_1838	marR	1.75	−1.38	−1.02	-	73	GTCAACTAAATGAAT	9.50E-05	DNA-binding transcriptional repressor MarR
STM1586	STM14_1918	-	5.55	2.75	1.06	+	171	GTTAAGAAAATGTGC	9.50E-05	putative periplasmic protein
STM3216	STM14_3893	tsr	1.17	−1.65	−1.31	-	198	GTTAACCATTTCTTA	8.10E-06	putative methyl-accepting chemotaxis protein
STM2445	STM14_3003	ucpA	−5.34	−1.83	−1.86	+	44	GTTAATGGAGTGTAA	1.20E-05	short chain dehydrogenase
STM1795	STM14_2170	gluD	−6.28	−1.34	−1.94	-	121	GTTAACTATCCGCTA	9.50E-05	putative glutamic dehydrogenase-like protein
STM4087	STM14_4913	glpF	−1.63	−5.7	8.77	+	217	GTTAATGAAATGATT	1.00E-05	glycerol diffusion
STM1771	STM14_2141	chaA	−1.77	−5.97	3.31	-	36	GTTAATATTTTGGAA	8.00E-05	calcium/sodium:proton antiporter
STM1125	STM14_1281	putP	−8.81	−3.4	−3.46	+	234	GTTAACACTTTTAAA	9.50E-05	major sodium/proline symporter
STM1091	STM14_1237	sopB	−2.17	−3.57	−5.89	+	52	GTTAACCCTGTTGAA	8.00E-05	secreted effector protein
STM2866	STM14_3463	sprB	−6.06	−2.32	−3.51	+	281	GTTAATGAAAGGGAA	8.10E-06	transcriptional regulator
STM4405	STM14_5290	ytfJ	−3.2	−1.45	−2.31	-	67	GTTAATCATATGTGC	3.30E-05	putative transcriptional regulator
STM4535	STM14_5449	-	−2.7	−1.04	−2.76	-	98	GTTAACAGAGGGAAA	9.50E-05	putative PTS permease
STM4467	STM14_5361		−1.83	1.02	−2.07	-	271	GTTAATTATTTGTTT	6.50E-06	arginine deiminase
STM1130	STM14_1293	nanM	−2.58	1.01	−2.92	+	115	GATAACTCCATGTAA	8.00E-05	putative inner membrane protein
STM4165	STM14_5006	rsd	2.75	1.69	2.74	-	67	GTTAACAACATGCCA	1.20E-05	anti-RNA polymerase sigma 70 factor
STM1728	STM14_2091	yciG	1.68	1.95	−2.11	-	261	GTTAATGCATTGTTT	1.50E-05	putative cytoplasmic protein
STM0292	STM14_0341	-	1.18	2.12	2.18	-	248	GTTCATCAAATGTAG	6.80E-05	putative RHS-like protein
STM2220	STM14_2744	yejG	5.84	2.41	2.94	+	64	GTCAATGATGTGTTA	6.80E-05	hypothetical protein
STM1770	STM14_2140	chaB	2.07	3.64	−2.12	+	245	GTTAATATTTTGGAA	8.00E-05	cation transport regulator
STM1211	STM14_1385.J	ndh	7.09	3.58	2.24	-	44	GTTAATTAAAAGTTA	1.10E-06	respiratory NADH dehydrogenase 2
						+	65	GTTAATTAAAGGCTA	1.00E-05	
						-	33	ATTAACCAATTGTTA	9.50E-05	
STM1746.S	STM14_2110	oppA	−7.87	−2.46	−3.08	+	318	GTTAACAAAATTGTA	1.00E-05	oligopeptide transport protein
						-	327	GTTAACCAATTCTCT	6.80E-05	
STM1818	STM14_2199	fadD	−1.56	−1.85	2.56	+	75	GTTAATATAATGTTA	1.00E-05	long-chain-fatty-acid–CoA ligase
						+	64	GTTAACGACTTGTTT	1.00E-05	
STM3692	STM14_4451	lldP	−13.27	−2.11	−6.83	-	125	GTTAACCAGATGTTA	2.00E-06	L-lactate permease
						+	136	GTTAACTATTTGTTG	5.20E-06	
						-	173	GTTAATTTAATGAAA	1.90E-05	
STM1303	STM14_1582	argD	−2.63	−1.11	−2.95	-	40	GTTATTTATATGTTA	2.80E-05	bifunctional succinylornithine transaminase
						+	112	GTTTATGCAATGTTA	5.70E-05	

[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].

Figure 3 Role of ArcA in modulating the redox status in aerobiosis and in response to H$_2$O$_2$. Strains 14028s and Δ*arcA* were grown to OD$_{600}$ ~ 0.4 and treated with 1.0 mM H$_2$O$_2$ for 20 min. Control cells received no treatment. The levels of **(A)** glutathione reductase (GR) activity, **(B)** reduced glutathione (GSH), oxidized glutathione (GSSG), **(C)** the GSH/GSSG ratio and **(D)** the NADH/NAD$^+$ ratio were measured. **(E)** Total ROS amount was measured under aerobic conditions. Values are the mean ± SD of three independent experiments. Values were normalized by protein concentration (A, B, C, D) or OD$_{600}$ **(E)**. AU: arbitrary units. GR activity: nmol NADPH min^{-1} ml^{-1} mg protein^{-1}. *p ≤ 0.05, **p ≤ 0.01, as compared to aerobically grown wild type cells.

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 H$_2$O$_2$ 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 H$_2$O$_2$ is provided in Additional file 2: Table S2.
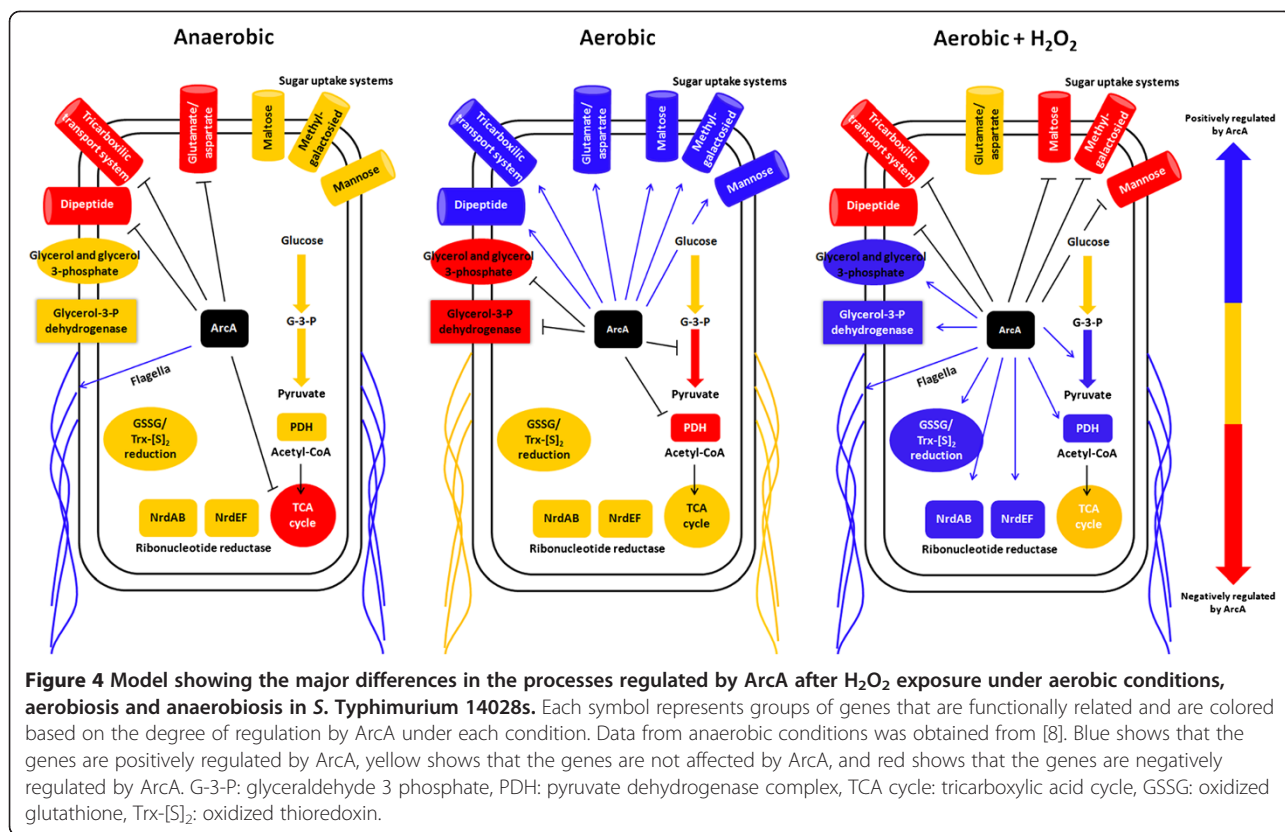
Role of ArcA in ROS scavenging

The *S*. Typhimurium genome codes for several genes that degrade H$_2$O$_2$ 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 H$_2$O$_2$, 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 H$_2$O$_2$ treatment. These results are in agreement with studies in *E. coli* that demonstrate that an *arcA* mutant does not show defects in H$_2$O$_2$ 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 H$_2$O$_2$, 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 Δ*arcA* (Additional

Figure 4 Model showing the major differences in the processes regulated by ArcA after H$_2$O$_2$ exposure under aerobic conditions, aerobiosis and anaerobiosis in S. Typhimurium 14028s. Each symbol represents groups of genes that are functionally related and are colored based on the degree of regulation by ArcA under each condition. Data from anaerobic conditions was obtained from [8]. Blue shows that the genes are positively regulated by ArcA, yellow shows that the genes are not affected by ArcA, and red shows that the genes are negatively regulated by ArcA. G-3-P: glyceraldehyde 3 phosphate, PDH: pyruvate dehydrogenase complex, TCA cycle: tricarboxylic acid cycle, GSSG: oxidized glutathione, Trx-[S]$_2$: oxidized thioredoxin.

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* Δ*gor* mutant has a slight sensitivity towards paraquat and cumene hydroperoxide [52], while in stationary phase a Δ*trxB* strain shows H$_2$O$_2$ sensitivity [53]. However, a double Δ*gor* Δ*trxB* 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 Δ*arcA* mutant treated with H$_2$O$_2$ 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 H$_2$O$_2$, 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 Δ*arcA* 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 Δ*arcA* mutant treated with H$_2$O$_2$ under aerobic conditions were purine and pyrimidine metabolism (Figure 4, Table 1). The major differences are found in the expression of the *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 H$_2$O$_2$ exposure, in agreement with studies in *E. coli* and *S.* Typhimurium 4/74 [41,43], while the regulation was lost in the Δ*arcA* mutant under the same conditions (Additional file 2: Table S2). NrdEF is usually repressed by Fur. However, in response to H$_2$O$_2$, this Fur repression is abolished and the apoprotein form of IscR upregulates expression of the operon [44]. This suggests that in response to H$_2$O$_2$ 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 $\Delta arcA$ 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 $\Delta arcA$ strain. Two studies conducted in *E. coli* measured the flux through the PDH complex in a $\Delta arcA$ mutant under aerobic conditions with different results. One showed that there was an increase in the flux through the PDH complex [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. Our analysis showed that the NADH/NAD$^+$ ratio was 2-fold higher in the aerobically grown $\Delta arcA$ mutant than in the wild type strain (Figure 3D). After H_2O_2 exposure, the NADH/NAD$^+$ ratio decreased in the wild type and $\Delta arcA$ strain, but in the latter the levels remained higher than in the wild type strain under aerobic conditions (Figure 3D). Since NADH can reduce Fe^{3+} to Fe^{2+} *in vitro* [56], and elevated NADH levels result in increased sensitivity towards H_2O_2 [58], the higher basal levels of NADH in the $\Delta arcA$ mutant in aerobiosis and after H_2O_2 treatment may increase Fe^{+2} turnover, fueling the Fenton reaction (the formation of OH·, and Fe^{3+} from the nonenzymatic reaction of Fe^{2+} with H_2O_2) and leading to higher levels of ROS-derived damage.

In the respiratory chain, NADH dehydrogenase II (encoded by *ndh*) generates O_2^- and H_2O_2 by oxidation of its reduced $FADH_2$ cofactor [58]. In an aerobically grown $\Delta arcA$ 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 $\Delta arcA$ 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 H_2O_2. However, several other sources of intracellular ROS besides NADH dehydrogenase II may also contribute to the higher levels of ROS observed in the $\Delta arcA$ mutant, such as fumarate-reducing flavoenzymes [59].

Conclusion

We identified the ArcA regulon in *S.* Typhimurium under aerobic growth with and without H_2O_2, and show that ArcA coordinates a response that includes changes in cellular-, glutathione-, thioredoxin-, NADH- and glycerolipid metabolism. These changes contribute to H_2O_2 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, $\Delta arcA$, $\Delta arcA::cat$/pBR::*arcA*, and $\Delta arcA::cat$/pBR::*arcA*D54A, after H_2O_2 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 H_2O_2 in the different genetic backgrounds.

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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