Hypochlorous acid and hydrogen peroxide-induced negative regulation of Salmonella enterica serovar Typhimurium ompW by the response regulator ArcA

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Abstract

Background: Hydrogen peroxide (H₂O₂) and hypochlorous acid (HOCl) are reactive oxygen species that are part of the oxidative burst encountered by Salmonella enterica serovar Typhimurium (S. Typhimurium) upon internalization by phagocytic cells. In order to survive, bacteria must sense these signals and modulate gene expression. Growing evidence indicates that the ArcAB two component system plays a role in the resistance to reactive oxygen species. We investigated the influx of H₂O₂ and HOCl through OmpW and the role of ArcAB in modulating its expression after exposure to both toxic compounds in S. Typhimurium.

Results: H₂O₂ and HOCl influx was determined both in vitro and in vivo. A S. Typhimurium ompW mutant strain (ΔompW) exposed to sub-lethal levels of H₂O₂ and HOCl showed a decreased influx of both compounds as compared to a wild type strain. Further evidence of H₂O₂ and HOCl diffusion through OmpW was obtained by using reconstituted proteoliposomes. We hypothesized that ompW expression should be negatively regulated upon exposure to H₂O₂ and HOCl to better exclude these compounds from the cell. As expected, qRT-PCR showed a negative regulation in a wild type strain treated with sub-lethal concentrations of these compounds. A bioinformatic analysis in search for potential negative regulators predicted the presence of three ArcA binding sites at the ompW promoter region. By electrophoretic mobility shift assay (EMSA) and using transcriptional fusions we demonstrated an interaction between ArcA and one site at the ompW promoter region. Moreover, qRT-PCR showed that the negative regulation observed in the wild type strain was lost in an arcA and in arcB mutant strains.

Conclusions: OmpW allows the influx of H₂O₂ and HOCl and is negatively regulated by ArcA by direct interaction with the ompW promoter region upon exposure to both toxic compounds.

Background

Hydrogen peroxide (H₂O₂) and hypochlorous acid (HOCl) are reactive oxygen species that are part of the oxidative burst encountered by S. Typhimurium upon internalization by phagocytic cells. Under acidic conditions, such as those found inside the phagosome, H₂O₂ is generated spontaneously by the reaction of two superoxide anion (O₂⁻) molecules [1]. Moreover, S. Typhimurium encodes both periplasmic and cytoplasmic superoxide dismutases that catalyze O₂⁻ dismutation to generate H₂O₂ and molecular oxygen [2-4]. HOCl is produced by the action of myeloperoxidase (MPO) in a reaction that depends on H₂O₂, Cl⁻ and acidic conditions [5,6]. Taken together, H₂O₂ and HOCl react with thiol and heme groups, copper and iron salts generating the reactive hydroxyl radical (OH·). As a consequence, they produce lipid peroxidation, chlorination of tyrosine residues, oxidation of iron centers, protein cross linking and DNA damage [5-8].

In order to enter Gram negative bacteria, H₂O₂ and HOCl must be able to cross the outer membrane (OM) and even though several biological membranes are
permeable to H$_2$O$_2$, studies in *E. coli* and *Saccharomyces cerevisiae* showed that this compound cannot diffuse freely [9,10]. For HOCl, diffusion through the OM is reported to be limited [11]. One possibility for H$_2$O$_2$ and HOCl influx through the OM is diffusion through porins. In this context, we recently reported that OmpD, *S. Typhimurium* most abundant OM porin, allows H$_2$O$_2$ diffusion [12]. OM porins are organized as homo-trimers (classic porins) or monomers (small porins) forming aqueous channels that allow the influx of hydrophilic solutes with a molecular weight ≤ 600 Da [13]. Classic porins, including OmpC and OmpF, form β-barrels with 12–22 transmembrane segments while small porins (OmpW) are composed of 8–10 [14,15]. The crystal structure of OmpW from *E. coli* revealed that it forms an 8-stranded β-barrel and functions as an ion channel in lipid bilayers [16,17]. In *Vibrio cholerae*, OmpW was described as an immunogenic 22 KDa protein [18] and its expression is altered by factors such as temperature, salinity, nutrient availability and oxygen levels [19]. Additionally, several studies show that porins are regulated by ROS. Due its oxidant nature and diffusion through the OM, regulation of porin expression must be tightly regulated as a mechanism of controlling OM permeability. Accordingly, *S. Typhimurium* ompD and ompW expression is regulated in response to H$_2$O$_2$ and paraquat [12,20], respectively, and *S. Enteritidis* and Typhimurium exposure to HOCl results in lower levels of ompD, ompC and ompF transcripts [21].

The cellular response to oxidative stress is regulated at the transcriptional level by activating the SoxRS and OxyR regulons in response to O$_2$ and H$_2$O$_2$, respectively [22,23], however, several studies have provided evidence for a role of the ArcAB two component system in the resistance to ROS induced damage [12,24–26]. ArcA is essential for *S. Enteritidis*, *Typhimurium* and *E. coli* resistance to ROS [24,26,27]. ArcB is a sensor member of the histidine kinase family that is anchored to the inner membrane [28]. In response to oxygen availability, ArcB autophosphorylates in an ATP dependent intramolecular reaction at position His-292 [29,30] and transfers the phosphate group to the cytoplasmic response regulator ArcA [31–33], which binds to promoter regions regulating gene expression [34,35]. ArcB activity is regulated in response to oxygen conditions by the redox state of both the ubiquinone and menaquinone pools [29,36–38]. However, recent studies in *E. coli* show that the system is regulated by the degree of aerobiosis but not by the redox state of the ubiquinone pool, challenging the idea that the system is inhibited by oxidized quinones [39].

In this work we provide further evidence of the role of the ArcAB two component system in the response to ROS under aerobic conditions and show that this system mediates regulation of *ompW* expression in response to a novel signal, HOCl. First we demonstrate, both in *vivo* and in *vitro*, that OmpW mediates diffusion of H$_2$O$_2$ and HOCl and that exposure of *S. Typhimurium* to these compounds results in a negative regulation of *ompW*. By EMSA and using transcriptional fusions, we demonstrate that the global regulator ArcA binds to the *ompW* promoter region. Furthermore, we show that *ompW* negative regulation observed in wild type cells treated with H$_2$O$_2$ and HOCl was not retained in an arcA or arcB mutant strain, indicating that the ArcAB two component system mediates *ompW* negative regulation in response to H$_2$O$_2$ and HOCl. These results further expand our knowledge in both the mechanisms of ROS resistance and the role of ArcAB in this process.

**Results and discussion**

The OmpW porin facilitates H$_2$O$_2$ and HOCl diffusion through the OM and reconstituted proteoliposomes

Hydrogen peroxide and hypochlorous acid are ROS generated by phagocytic cells and in order to enter Gram-negative bacteria they must be able to cross the OM. Even though several biological membranes are permeable to H$_2$O$_2$, studies in *E. coli* and *S. cerevisiae* demonstrate that this compound cannot diffuse freely [9,10]. Additionally, the dielectric properties of H$_2$O$_2$ are comparable to those of water and this compound has a slightly larger dipolar moment, further limiting its diffusion through the OM lipid bilayer. For HOCl, diffusion through the OM is also reported to be limited [11]. Therefore, H$_2$O$_2$ and HOCl must be channeled through the lipid bilayer and one possibility is the influx through porins. We recently demonstrated that the most abundant OM protein in *S. Typhimurium*, OmpD, allows H$_2$O$_2$ diffusion and is regulated by ArcAB [12]. Little is known about the diffusion of HOCl, but genetic evidence has suggested that in *E. coli* porins might be used as entry channels for hypoiodoacetate ions (OSCNO$^-$), a molecule with a similar chemical structure generated by lactoperoxidase using thiocyanate and H$_2$O$_2$ as an oxidant [40]. In one study, ompC and ompF knockout mutants showed an increased resistance to OSCNO$^-$, however, a direct role of porins in mediating HOCl diffusion was not evaluated.

To assess whether OmpW allows the diffusion of H$_2$O$_2$ and HOCl, scopoletin and dihydrorhodamine (DHR)-123 probes, respectively, were used to measure uptake of both toxic compounds separately in a wild type, Δ*ompW* and a genetically complemented Δ*ompW* (pBAD-ompW) strain as described in methods. The Δ*ompW* strain showed an increase in extracellular fluorescence levels after exposure to H$_2$O$_2$ and HOCl resulting in higher extra/intracellular ratios (24 and 4-fold, respectively) as compared to the wild type strain, indicating that in the absence of OmpW the influx of both toxic compounds is decreased. Genetic complementation of Δ*ompW* resulted in nearly identical
levels of both extra and intracellular fluorescence as those observed in the wild type strain, suggesting that OmpW is necessary for H$_2$O$_2$ and HOCl uptake (Figure 1A and C). Even though OmpW appears as a direct responsible for the influx of the compounds, a pleiotropic effect cannot be ruled out at this point because the absence of OmpW in the mutant strain could be producing a remodeling of the membrane organization.

To establish a direct contribution of OmpW in H$_2$O$_2$ and HOCl transport, we used reconstituted proteoliposomes. OmpW-proteoliposomes showed a decrease in H$_2$O$_2$ and HOCl extra/intraliposomal ratios (3.5 and 5-fold respectively) when compared to free liposomes (Figure 1B and D). Proteoliposomes with S. Typhimurium OmpA porin were used as a negative control as previously described [12]. As expected, OmpA-proteoliposomes showed similar levels to those of free liposomes, indicating that OmpW facilitates H$_2$O$_2$ and HOCl uptake.

Since OmpW channels both toxic compounds across the lipid bilayer, we hypothesized that a ΔompW strain should be more resistant to both toxic compounds when compared to the wild type strain. As shown in Figure 2, exposure of ΔompW to H$_2$O$_2$ 4 mM or HOCl 5 mM resulted in an increase in the number of colony forming units (CFU) after 60 min of treatment. However, at longer periods the CFU count between strains 14028s and ΔompW was similar. At 30 min post-treatment with either of the toxic compounds, strain ΔompW showed an increase from 1×10^6 CFU/ml to approximately 6×10^7 CFU/ml. In contrast, the CFU/ml count for strain 14028s remained almost unaltered at 1×10^6, resulting in a 1.5-log$_{10}$-fold increase in growth for ΔompW. A similar result was observed after 60 min of treatment where the ompW mutant strain showed an increase from 6×10^7 to 1.5×10^9 CFU/ml while the wild type strain changed from 1×10^6 to 8×10^7 CFU/ml. Our results suggest that the absence of OmpW in the mutant strain represents an advantage at short time points due to a decreased permeability towards both H$_2$O$_2$ and HOCl. At longer periods, OM permeability should be reduced because exposure to both toxic compounds results in a negative regulation of S. Typhimurium porins including OmpD, OmpC and OmpF [12,21]. One important possibility that cannot be ruled out at this time is that in the ΔompW strain, the expression of other porins or the OM lipid composition might be altered, therefore changing OM

![Figure 1](http://www.biomedcentral.com/1471-2180/12/63)
permeability. For example, a study conducted in *E. coli* showed that an *ompC* knockout mutant had increased levels of OmpA [40], however, changes in permeability were not evaluated. Furthermore, this has not been evaluated in a *S. Typhimurium* or *E. coli* Δ*ompW* strain.

Our data supports the proposed model where OmpW allows the influx of small polar molecules, like H2O2 and HOCl. The crystal structure of OmpW from *E. coli* revealed that the cross-section of the barrel has approximate dimensions of 17 × 12 Å along the length of the barrel and although the interior of the channel has a hydrophobic character, the observed single channel activities show that polar molecules traverse the barrel [17]. Taken together, these results provide biochemical and genetic evidence indicating that both toxic compounds are channeled through OmpW. From our knowledge, this is the first direct evidence of HOCl diffusion through porins. Furthermore, preliminary analyses indicate that H2O2 and HOCl channeling is common for *S. Typhimurium* OmpD, OmpC and OmpF porins (unpublished data).

**Hydrogen peroxide and hypochlorous acid exposure results in *ompW* negative regulation**

Since the OmpW porin channels H2O2 and HOCl through the OM and exposure to these molecules is detrimental to bacteria, we hypothesized that *ompW* should be negatively regulated when *S. Typhimurium* is exposed to H2O2 and HOCl. To study this effect, wild type *S. Typhimurium* cells were grown to mid-log phase, exposed to H2O2 or HOCl and *ompW* mRNA levels were measured by qRT-PCR. As seen in Figure 3, exposure to H2O2 and HOCl resulted in lower levels of *ompW* transcripts (0.27 ± 0.04 and 0.156 ± 0.079, respectively) relative to control untreated cells. In agreement with our results of *ompW* negative regulation, similar results were observed by Wang et al. (2010) who showed that *S. Enteritidis* and *Typhimurium* cells exposed to HOCl results in modulation of *ompD*, *ompC*, *ompF* (negatively) and *ompA* (positively) expression. Furthermore, Calderón et al. (2011) demonstrated that the *S. Typhimurium* *ompD* gene is negatively regulated in response to H2O2. Therefore, our and all the published data suggest that in the presence of OCl- or H2O2 there might be a general lowering in the concentration of porins in the outer membrane, in order to diminish the permeability.

To assess the specificity of our assay, we evaluated *ompD*, *ompC* and arcB transcript levels as positive (*ompD* and *ompC*) and negative controls (*arcB*). The *arcB* gene was used as a negative control based on our microarray analysis which shows that it remains unaltered under these conditions and between strains 1408s and ΔarcA (unpublished data). Our results indicate that after exposure to both toxic compounds, arcB transcript levels remain unchanged while those of *ompD* and *ompC* are lowered as compared to untreated cells (Figure 3). Therefore, all the evidence indicates that OM permeability is tightly regulated in response to ROS and could represent a novel mechanism of resistance when bacteria are exposed to these toxic compounds.

**ArcA binds the *ompW* promoter region**

In addition to the soxRS and oxyR systems, several studies have provided evidence that the ArcAB two component system plays an important role in the resistance to
ROS induced damage. For example, ArcA is essential for
S. Enteritidis and Typhimurium resistance to ROS
[24,27] and E. coli mutant strains of the sensor ArcB and
the regulator ArcA, show an increased susceptibility to
H₂O₂ [26]. However, neither of these studies identified
genes directly regulated by the system under oxidative
stress. We recently demonstrated that ArcA negatively
regulates the expression of S. Typhimurium ompD after
H₂O₂ exposure by direct interaction with its promoter
region [12]. To determine if ArcA mediates ompW
down-regulation in response to H₂O₂ and HOCl, a
search for putative ArcA binding sites at the ompW pro-
moter region was performed using Virtual Footprint 3.0
[41]. The analysis predicted the presence of three ArcA
binding sites (ABS) located at positions −61 to −70
(ABS-1, forward orientation), -230 to -239 (ABS-2) and
−286 to −295 (ABS-3, both in reverse orientation) rela-
tive to the experimentally determined transcription start
site [42]. Comparison with the extended core region 5′-
GTTAATTAAATGTTA-3′ described by Evans et al.
(2011) further revealed that only ABS-1 presented a high
degree of identity (14 out of 15 nucleotides) with the
consensus sequence. To confirm or rule out a direct
interaction between ArcA and the predicted binding
sites, deletions of the promoter region were generated by
PCR (schematized in Figure 4B) and used to perform
non-radioactive EMSAs with ArcA and phosphorylated
ArcA (ArcA-P). The purity of the protein was assessed by
PAGE and ArcA was the dominant product. Electrophor-
etic mobility shift with ArcA-P was only observed when
incubated with fragments that included ABS-1 (Figure 4C
and D, W1 and W4). No shifts were observed in fragments
that include both ABS-2 and ABS-3 (W3, even at three-
fold excess) or control fragments that did not include any
ABS (W2 and W5). Non-phosphorylated ArcA only gener-
ated electrophoretic mobility shifts at higher concen-
trations (over 1200 nM) where the negative controls were
also retarded as a result of non-specific binding (Figure 3E).
Taken together our bioinformatic and EMSA analyses in-
dicate that ArcA-P binds to the ompW promoter region
at a site located between positions −80 and −41 and suggests
that this site is ABS-1 which is located between positions
−70 to −55.

Evaluating ArcA binding site 1 (ABS-1) functionality
To further confirm that ABS-1 (Figure 4A) was the func-
tional ArcA binding site mediating ompW negative regu-
lation in response to ROS, we constructed transcrip-
tional fusions of the ompW promoter region.
We generated two different fusions which included the
whole promoter from positions +1 to −600, with respect
to the translation start site. One construction contained
the native promoter (pompW-lacZ) while substitutions
that mutated ABS-1 (shown in red and underlined,
Figure 5A) were included in the second construction (pompW/ABS1-lacZ). The constructions were transformed into the wild type strain and β-galactosidase activity was measured in response to treatment with H₂O₂ and HOCl.

The activity of the constructions was compared to the untreated 14028s strain with the wild type fusion. Treatment of this strain with H₂O₂ and HOCl resulted in lower activity levels (0.58 ± 0.008 and 0.53 ± 0.095, respectively), in agreement with qRT-PCR experiments. However, a 5 nucleotide substitution of the most conserved residues at ABS-1 site (pompW/ABS1-lacZ) resulted in no regulation after exposure to either of the toxic compounds (1.09 ± 0.104 and 0.93 ± 0.061), indicating that they are relevant for the transcriptional activity of ompW in response to H₂O₂ and HOCl (Figure 5B). Furthermore, these results are in agreement with EMSAs which indicate that ArcA only binds to fragments containing ABS-1.

The ArcAB two component system mediates ompW negative regulation

To establish a direct relationship between ompW negative regulation and ArcA-P binding to its promoter region, ompW expression was evaluated by qRT-PCR in a ΔarcA strain exposed to H₂O₂ and HOCl. The negative regulation observed in the wild type strain was not retained in an arcA mutant treated with either of the toxic compounds and ompW transcript levels were similar as those observed in untreated cells. Genetic complementation of ΔarcA restored the negative regulation observed in wild type cells exhibiting lower ompW mRNA levels (0.161 ± 0.068 and 0.488 ± 0.027, respectively) as compared to untreated cells (Figure 6A and C). Growth of the genetically complemented strain in the presence of glucose (non-induction) resulted in similar ompW mRNA levels between treated and untreated cells (data not shown). As controls, we measured ompD, ompC and arcB transcript levels after exposure to H₂O₂ and HOCl in a ΔarcA strain. Transcript levels of ompD were measured since its expression is regulated by ArcA under ROS conditions [12]. Our results indicate that neither ompD or arcB transcript levels were decreased after exposure to H₂O₂ or HOCl while those of ompC remained regulated in a ΔarcA strain treated with either of the toxic compounds (Figure 6A), confirming that ArcA mediates ompD regulation under ROS conditions and showing that the expression of ompC is ArcA independent and regulated by different mechanisms which remain unsolved to the date, and are under study in our laboratory. Furthermore, our bioinformatic analyses in search for ArcA motifs predicted binding sites in the promoter regions of ompW and ompD, but not for ompC ([12], data not shown).

To determine whether the negative regulation by ArcA was dependant on its cognate sensor ArcB, ompW expression was evaluated by qRT-PCR in a ΔarcB strain exposed to H₂O₂ and HOCl. The negative regulation observed in the wild type strain was not retained in an arcB mutant treated with either of the toxic compounds and ompW transcript levels were similar as those observed in untreated cells. Genetic complementation of ΔarcB restored the negative regulation observed in wild type cells exhibiting lower ompW mRNA levels (0.161 ± 0.068 and 0.488 ± 0.027, respectively) as compared to untreated cells (Figure 6A and C). Growth of the genetically complemented strain in the presence of glucose (non-induction) resulted in similar ompW mRNA levels between treated and untreated cells (data not shown). As controls, we measured ompD, ompC and arcB transcript levels after exposure to H₂O₂ and HOCl in a ΔarcB strain. Transcript levels of ompD were measured since its expression is regulated by ArcA under ROS conditions [12]. Our results indicate that neither ompD or arcB transcript levels were decreased after exposure to H₂O₂ or HOCl while those of ompC remained regulated in a ΔarcB strain treated with either of the toxic compounds (Figure 6A), confirming that ArcA mediates ompD regulation under ROS conditions and showing that the expression of ompC is ArcA independent and regulated by different mechanisms which remain unsolved to the date, and are under study in our laboratory. Furthermore, our bioinformatic analyses in search for ArcA motifs predicted binding sites in the promoter regions of ompW and ompD, but not for ompC ([12], data not shown).

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mRNA levels were evaluated in a ΔarcB strain. In contrast to the negative regulation observed in wild type cells, ompW mRNA levels were further increased in a ΔarcB strain after exposure to HOCl (3.37 ± 1.09). Transcript levels after treatment with H2O2 were similar as those observed in untreated cells (Figure 6B). One possibility for this result is that in the absence of ArcA, ArcB might phosphorylate (i.e ArcB-OmpR, [43]) one or more response regulators, either unspecifically or due to cross-talk, which could bind to the promoter region and therefore prevent binding of positive regulators like SoxS, which has been demonstrated to regulate ompW and is up-regulated in response to HOCI [20,44]. This could result in constant ompW transcript levels as shown in Figure 6A. On the other hand, in the absence of ArcB no phosphorylation occurs and SoxS or other positive regulator(s) might have free accessibility to the ompW promoter and therefore increase its expression (Figure 6B), although this possibility has not been evaluated in this study. Genetic complementation of ΔarcB restored the negative regulation observed in wild type cells exposed to H2O2 and HOCI (0.19 ± 0.04 and 0.24 ± 0.11, respectively, Figure 6C). The ompD and ompC transcripts levels remained down-regulated after exposure to H2O2 and HOCI in the ΔarcB strain, while the negative control arcA remained unaltered (Figure 6B).

The ArcA regulon in anaerobically grown S. Typhimurium was recently determined [27]. Interestingly, neither ompD nor ompW expression was down-regulated in an ArcA dependant manner, suggesting that the ArcA regulon under anaerobic and aerobic ROS conditions could be different. Even in E. coli, ompW expression is suggested to be regulated by FNR in response to oxygen availability [39]. The difference between the ArcA regulons under aerobic and ROS conditions might be explained by studies suggesting that the mechanism of ArcA activation under aerobic conditions is different from those classically described. E. coli mutant strains in residue H-717 of ArcB are able to phosphorylate and activate ArcA through the transfer of the phosphate group from residue His-292 under aerobic conditions [45] and Loui et al. (2009) suggested that H2O2 resistance is independent of ArcA phosphorylation at residue Asp-54. To the date, the detailed molecular mechanism of ArcAB activation in response to ROS remains unsolved. Therefore, further experiments to unveil the molecular mechanism by which the S. Typhimurium ArcAB two component system is activated are needed and under way in our laboratory.

**Conclusion**

We provide both genetic and biochemical evidence indicating that the OM porin OmpW mediates the influx of H2O2 and HOCI. The results revealed that the S. Typhimurium ompW gene is negatively regulated upon exposure to both toxic compounds. Furthermore, we demonstrate that the response regulator ArcA mediates ompW negative regulation in response to H2O2 and HOCI via a direct interaction with the upstream region of ompW. Taken together, with our previous observation that OmpD mediates influx of H2O2 and is negatively regulated by ArcA in response...
to H₂O₂, these results further expand our knowledge regarding the coordinated regulatory mechanisms of ROS resistance and the role of ArcAB in this process.

Methods

Bacterial strains and growth conditions

Bacterial strains used in this work are listed in Table 1. Cells were grown aerobically with agitation in LB medium at 37°C. Solid media consisted of agar (20 g l⁻¹) and plates were incubated at 37°C. Dilutions (1:100) of overnight cultures were used to initiate growth. When necessary, growth media was supplemented with the appropriate antibiotics (see below).

Strain construction and genetic complementation

S. Typhimurium arcB gene was interrupted by gene disruption as previously described [46]. Strain 14028s (wild type) harboring plasmid pKD46 was grown in the presence of arabinose (10 mM) and ampicillin (100 μg ml⁻¹) to OD₆₀₀ ~ 0.4, made electrocompetent and transformed with a PCR product generated with plasmid pKD3 as template and primers 5’ ATTGGGTATTATGTGGAAGTTGTGGTGAAGGAATCCTCTTGTAGGCTGGA GCTGCTTCG 3’ (WarcBF) and 5’ GGTGTTGGCAGTATTCGGACCCCGGTCAACCGGGCATATGAA-TATCCTTCTTAG 3’ (WarcBR). Transformants were selected on LB plates supplemented with chloramphenicol (20 μg ml⁻¹) and confirmed by PCR using primers 5’ ATGAAGCAAATTCGTATGCTG 3’ (pBADarcBF) and 5’ TCATTTTTTTTCCGCGTTTGC-CACCC 3’ (pBADarcBR) and cloned into plasmid pBAD-TOPO TA® (Invitrogen) according to manufacturer’s instructions. Insertion was verified by DNA sequencing.

Bacterial survival after exposure to oxidative stress

Bacteria were cultured in 5 ml of LB medium at 37°C overnight with shaking. Antibiotics were added as appropriate. 1:1000 dilutions of the overnight cultures were grown in 25 ml to OD ~ 0.4 and H₂O₂ 4 mM or NaOCl 5 mM (final concentration) were added. In all the assays the cultures were grown aerobically at 250 rpm. Aliquots

Table 1 Bacterial strains used in this study

| Strain                        | Relevant characteristic(s) | Source                        |
|-------------------------------|-----------------------------|-------------------------------|
| S. Typhimurium                |                             |                               |
| 14028s                        | wild type strain            | G. Mota                       |
| 14028s/pompW-lacZ             | 14028s transformed with a derivative of plasmid pLacZ-Basic carrying the ompW promoter (nt -600 to +1) | This work                     |
| 14028s/pompW/ABS1-lacZ        | 14028s transformed with a derivative of plasmid pLacZ-Basic carrying the ompW promoter (nt -600 to +1) with substitution GTTAA to TCCGG into position -70 to -66 | This work                     |
| ΔompW                         | ompW:kan                    | C. Saavedra                   |
| ΔompW/pBAD-ompW               | ΔompW strain complemented with pBAD vector carrying the S. Typhimurium ompW gene | C. Saavedra                   |
| ΔarcA                         | arcA:cam                    | [12]                          |
| ΔarcA/pBAD-arcA               | ΔarcA strain complemented with pBAD vector carrying the S. Typhimurium arcA gene | [12]                          |
| ΔarcB/pBAD-arcB               | ΔarcB strain complemented with pBAD vector carrying the S. Typhimurium arcB gene | This work                     |
| E. coli Top10                 | F- mcrA Δ(mcr-hsdRMS-mcrBC) Δ800lacZΔM15 ΔlacX74 recA1 araD139 Δ(ara-leu)7697 galK rpsL (StrR) endA1 nupG | Invitrogen                    |
| Top10 pBAD-ompW               | Top10 transformed with the pBAD vector carrying the S. Typhimurium ompW gene |                               |
| Top10 pBAD-ompA                | Top10 transformed with the pBAD vector carrying the S. Typhimurium ompA gene | C. Saavedra                   |
| Top10 pBAD-arcB                | Top10 transformed with the pBAD vector carrying the S. Typhimurium arcB gene | This work                     |
| BL21 pET-TOPOArcA             | BL21(DE3) transformed with the pET-TOPO101ArcA vector carrying the S. Typhimurium arcA gene | [12]                          |
of cultures were withdrawn at the different time points, diluted and plated in triplicate. Bacterial cultures were enumerated by counting the number of CFU after overnight incubation to determine the bacterial concentrations.

Construction of transcriptional fusions with reporter gene $lacZ$

The native $ompW$ promoter region from positions +1 to −600 (with respect to the translation start) site was amplified by PCR with primers $ompW_{\text{pLacZ}}$−600F_ATG 5′ CGGGGTACCCCGATACTGGAATTGCAG 3′ and $ompW_{\text{pLacZ}}$−1R_ATG 5′ CCCAAGCTTACCGGCTCATCGTTATGCT 3′ using genomic DNA from S. Typhimurium (strain 14028s). The restriction sites (KpnI and HindIII, respectively) at the ends of the DNA fragment were introduced by the PCR primers (underlined sequences) and digested with the corresponding enzymes. The digested PCR product was cloned into the multiple cloning site (MCS) of the β-galactosidase reporter vector pLacZ-Basic (GenBank accession no. U13184), Clontech, generating plasmid pompW-lacZ. To generate plasmid pompW/ABS1-lacZ, primers ompW_pLacZ_−600F_ATG with Mut sit arcAR 5′ TGTTCTTATATATGGTGTCGAAATT-TATTGCCAG 3′ and ompW_pLacZ_−1R_ATG with Mut sit arcAR 5′ CTGGAATCATATATCCGGAAT-TATAAGAACA 3′ were used to generate overlapping PCR products spanning the whole length of the $ompW$ promoter. Mutation of ABS-1 was generated by incorporating substitutions in primers Mut sit arcAR and Mut sit arcAR (underlined sequences). The resulting PCR products were used as templates in a second reaction with primers $ompW_{\text{pLacZ}}$−600F_ATG and $ompW_{\text{pLacZ}}$−1R_ATG to generate the mutated $ompW$ promoter, which was digested and cloned into the MCS of plasmid pLacZ-Basic. Constructions were confirmed by DNA sequencing. The generated constructs were transformed into wild type strain 14028s. To evaluate activity, cells at OD$_{600}$ ~ 0.4 were grown for 20 min in the presence of H$_2$O$_2$ (1.5 mM) or NaOCl (530 μM). Control cells received no treatment. After exposure to the toxic compounds, 4 ml were withdrawn from the culture and used to extract total RNA using GenElute Total RNA purification Kit® (Sigma). Total RNA treatment with DNase I and cDNA synthesis was performed as previously described [19].

Relative quantification of $ompW$ mRNA was performed using Brilliant II SYBR Green QPCR Master Reagent Kit and the Mx3000P detection system (Stratagene). 16S rRNA was used for normalization. Specific primers were 5′ ATGAAAAAAATTACAGTGCG 3′ (RTompWF) and 5′ GAAACGATAGCCTGCGG 3′ (RTompWR) for the $ompW$ gene; 5′ GTAGAATTCAGGTAGCCGCGC 3′ (16SF) and 5′ TTATCTGCGCTCTTCTC 3′ (16SR) for 16S rRNA gene (16S). 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 10 x Master Mix, 0.14 μl of diluted ROX (1:200) and 8.38 μl of H$_2$O. 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 53°C and 45 s at 72°C. Finally a melting cycle from 53 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 [47]. Experiments were performed in three biological and technical replicates.

DNA binding assays

Non-radioactive EMSAs were performed as described [48]. Briefly, increasing amounts of purified ArcA (phosphorylated and unphosphorylated) were incubated...
with 20 or 60 ng of PCR product(s) in binding buffer (100 mM Tris-Cl [pH 7.4], 100 mM KCl, 10 mM MgCl₂, 10% glycerol, and 2 mM dithiothreitol) for 20 min at 30°C. Reaction mixtures were immediately loaded on prerun 4% native polyacrylamide gels. The DNA-protein complexes were visualized by ethidium bromide staining. PCR fragments used in EMSAs were generated by PCR using reverse primer 5′ ACCTGCTCATCCTGTATGT 3′ (ompWR) in combination with 5′ GAGCAGAAAAATTTGCGAT 3′ (300WF) or 5′ TATTAGATCATTATTACTT 3′ (170WF) to generate fragments W1 and W2, respectively. Fragment W3 was generated using primers 300WF and 5′ GATCCA-GATTAATTAGAAC 3′. Fragments W4 and W5 were generated by using reverse primer 5′ AATTTTTTATC TACCGTCTCC 3′ in combination with primers 5′ CCTATAAACCAGATTTTCAA 3′ and 170WF, respectively. ArcA phosphorylation was carried out as described [19] and was visualized by ethidium bromide staining. PCR fragments were visualized by ethidium bromide staining. EMSAs were generated by using reverse primer 5′ TATTAGATCATTATTACTT 3′ (170WF) to generate fragments W1 and W2, respectively. ArcA phosphorylation was carried out as described [19] and was visualized by ethidium bromide staining.

In vivo and in vitro determination of hydrogen peroxide and hypochlorous acid diffusion

In vivo diffusion of H₂O₂ was assessed as previously described [12]. For HOCl detection, overnight cultures were diluted and cells were grown to OD₆₀₀ ~ 0.5. Two ml of bacterial culture were centrifuged for 5 min at 4500 g and resuspended in 1 ml of 100 mM phosphate buffer (pH 7.2). A 200 μl aliquot was incubated for 5 min with 530 μM NaOCl and constant agitation. Following, cells were vacuum filtered using polycarbonate filters of 0.025 μM as previously described [49]. Bacteria retained in the filter were recovered with 1 ml of 50 mM disodium carbamoyl phosphate (Sigma) in a buffer containing 100 mM Tris-Cl (pH 7.4), 10 mM MgCl₂, 125 mM KCl, for 1 h at 30°C and used immediately in EMSA assays.

Fluorescence was measured in both fractions as described above and H₂O₂ or HOCl uptake was determined as the extraliposomal/intraliposomal fluorescence ratio.

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Author’s contributions

EHM and CPS conceived the project. EHM, BC and ILC performed the experiments. FG and SPJ conducted partial data analysis. EHM, ILC, MM and CPS wrote the paper. All authors read and approved the final manuscript.

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