Genetic regulation of theompX porin ofSalmonella Typhimurium in response to hydrogen peroxide stress

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Abstract

Background: Salmonella Typhimurium is a Gram-negative pathogen that causes a systemic disease in mice resembling typhoid fever. During its infective cycle, S. Typhimurium is phagocytized by macrophages and proliferates inside a Salmonella-containing vacuole where Salmonella is exposed and survives oxidative stress induced by H\textsubscript{2}O\textsubscript{2} through modulation of gene expression. After exposure of Salmonella to H\textsubscript{2}O\textsubscript{2}, the expression of the porin-encoding gene ompX increases, as previously shown by microarray analysis. Expression of ompX mRNA is regulated at a post-transcriptional level by MicA and CyaR sRNAs in aerobiosis. In addition, sequence analysis predicts a site for OxyS sRNA in ompX mRNA.

Results: In this work we sought to evaluate the transcriptional and post-transcriptional regulation of ompX under H\textsubscript{2}O\textsubscript{2} stress. We demonstrate that ompX expression is induced at the transcriptional level in S. Typhimurium under such conditions. Unexpectedly, an increase in ompX gene transcript and promoter activity after challenges with H\textsubscript{2}O\textsubscript{2} does not translate into increased protein levels in the wild-type strain, suggesting that ompX mRNA is also regulated at a post-transcriptional level, at least under oxidative stress. In silico gene sequence analysis predicted that sRNAs CyaR, MicA, and OxyS could regulate ompX mRNA levels. Using rifampicin to inhibit mRNA expression, we show that the sRNAs (MicA, CyaR and OxyS) and the sRNA:mRNA chaperone Hfq positively modulate ompX mRNA levels under H\textsubscript{2}O\textsubscript{2}-induced stress in Salmonella during the exponential growth phase in Lennox broth.

Conclusions: Our results demonstrate that ompX mRNA is regulated in response to H\textsubscript{2}O\textsubscript{2} by the sRNAs CyaR, MicA and OxyS in Salmonella Typhimurium.

Keywords: ompX, Translational regulation, Transcriptional regulation, H\textsubscript{2}O\textsubscript{2} stress

Introduction

Every year, Salmonella enterica causes around 1800 food-borne illness cases in the United States, resulting in about 200 hospitalizations. Most people develop diarrhea, fever, and abdominal pain 12 to 72 h after infection (CDC, Salmonella). During infection, Salmonella survives within innate immune host cells, including macrophages [37], where bacteria are exposed to adverse conditions that limit its survival. Reactive Oxygen Species (ROS) are the most harmful of these conditions [20]. ROS generated by phagocytic cells, particularly superoxide anions (O\textsubscript{2}\textsuperscript{−}) and hydrogen peroxide (H\textsubscript{2}O\textsubscript{2}), target bacterial components such as proteins, membranes, and nucleic acids [21].

During its infective cycle, internalized Salmonella faces H\textsubscript{2}O\textsubscript{2} and other toxic molecules that enter the bacterium...
through the outer membrane ([40], Faucher et al. 2006). Studies in Escherichia coli and Saccharomyces cerevisiae show that H2O2 cannot freely diffuse across membranes [41, 43]. Evidence from Salmonella Typhimurium (S. Typhimurium) indicates that ROS are channeled through porins such as OmpW and OmpD, which are down-regulated under oxidative stress in-vitro, highlighting their importance for survival [7, 31]. To survive the micro-environment inside the phagosome, Salmonella must finely-tune gene expression [16, 35, 36], including the expression of those genes that encode outer membrane proteins, which results in minimizing the influx of ROS [7, 10, 31]. However, a microarray analysis of the transcriptomic profile of S. Typhimurium has shown that the expression of the gene that codifies the porin ompX was 3.65 times greater under stress conditions compared to control settings [32], suggesting a role for higher level regulation during H2O2-induced stress for the modulation of the permeability of the outer membrane (Calderon et al. 2011, [32]).

OmpX is a small porin composed of eight antiparallel strands in a barrel conformation [47], that is involved in the responses to H2O2 stress, host invasion, iron homeostasis, and recognition of bacteria by the host’s adaptive immune response [9, 24, 25, 28, 29]. ompX gene expression is regulated at various levels; transcription is increased by overexpression of MarA in Enterobacter aerogenes [3, 14] and by H2O2 exposure in uropathogenic Escherichia coli [6].

Mecsas et al. [30] identified two promoters and a rho-terminator in the ompX gene in Escherichia coli. One of the promoters is bona fide, with sigma 70 boxes (50 bp upstream of the ATG of ompX gene). However the authors proposed that the other promoter (221 bp upstream of the ATG of ompX) is not activated by sigma 70 under the experimental conditions tested, and could require a supercoiled template, another form of the RNA polymerase or additional transcription factors to promote transcription. The authors suggest that expression of the ompX gene is induced under basic pH conditions via the second promoter (221 bp upstream of the ATG), but did not show in vivo evidence to demonstrate promoter activity.

At the post-transcriptional level, ompX is regulated by a group of non-coding small RNAs (sRNAs) which modulate the production of the protein and are implicated in a broad array of pathways, including carbon metabolism, iron homeostasis, quorum sensing, biofilm biosynthesis, and stress responses, among other functions [39]. Previous studies suggest that in Escherichia coli grown in rich media (Lennox broth), ompX mRNA is targeted by the sRNAs MicA and CyaR [22]. During oxidative stress conditions produced by H2O2, only OxyS sRNA was implicated in the post-transcriptional response, and the main mRNAs regulated by this OxyS encode for proteins required for oxidative stress resistance in Escherichia coli [1]. Additionally, the sRNAs CyaR, MicA and OxyS are regulated by the action of Hfq, a sRNA:mRNA chaperone, which stabilizes this interaction, promoting the degradation by RNAses or the translation of the transcript [22, 51], which is also required for regulation under diverse stress conditions [42].

In this work, we investigated ompX expression under hydrogen peroxide stress at the transcriptional and post-transcriptional level in S. Typhimurium 14028 s. Our results show that ompX transcript levels increase under peroxide stress, yet there are no changes at the protein level under the same conditions. Therefore, we hypothesize that MicA, CyaR and OxyS could play relevant roles in the regulation of the translation of ompX mRNA under peroxide stress.

**Materials and methods**

**Bacterial strains and growth conditions**

The bacterial strains and plasmids included in this study are listed in Additional file 1: Table S1. Bacteria were routinely grown in Lennox Broth (LB) at 37°C supplemented, when necessary, with ampicillin (0.1 mg/ml), chloramphenicol (0.02 mg/ml), or kanamycin (0.05 mg/ml). Cells grown to OD600 ≈ 0.4 were treated with 2 mM H2O2 in LB.

**Construction of chromosomal gene fusions with pSUB11 plasmid**

The ompX::3xflag strain was constructed as described by Uzzau et al. [45] by fusing the 3xflag epitope with the ompX gene. The primers were designed with 40 homology bases corresponding to the coding regions of the gene and a region immediately downstream to amplify the pSUB11 plasmid (Additional file 2: Table S2). The PCR products were used to transform electrocompetent 14028 s cells carrying plasmid pKD46. Fusion was confirmed by PCR. The 3xFlag-fusion protein was detected by immunoblotting using an anti-FLAG M2 monoclonal antibody (Sigma) and peroxidase-conjugated anti-mouse IgG (Sigma). Proteins were purified and detected as described elsewhere [18].

**Construction of a GFP-transcriptional fusion of ompX**

ompX promoter activity was evaluated by cloning the ompX promoter into the pGLO vector (Biorad). For this purpose, 375 bp of the ompX promoter were amplified by PCR (primers Prom_ompX_-1R and Prom_ompX_-375F, Additional file 2: Table S2), and the amplicon and pGLO plasmid were digested using the restriction enzymes BmiI and AgeI for 1 h at 37 °C. Products were purified
using the High Pure PCR Template Kit (Roche) following the manufacturer’s instructions. The PCR products were ligated to the digested pGLO plasmid using T4 ligase at 4 °C overnight. Electrocompetent E. coli TOP10 cells were then transformed with the resulting plasmid, denominated pGLO_ompX. The presence and orientation of the ompX promoter was verified by PCR, then the pGLO_ompX plasmid was purified using the High Pure Plasmid Isolation Kit (Roche) and transformed into electrocompetent S. Typhimurium 14028 s cells. Finally, strains carrying the pGLO_ompX and the pGLO plasmids were used to measure peroxide stress after 20 min of treatment.

Reporter activity
Strains carrying the plasmids pGLO and pGLO_ompX, were grown to OD$_{600}$ ≈ 0.4, centrifuged at 4400 rpm for 10 min to concentrate the cells, and suspended in 1 ml 20 mM 1X Phosphate Buffered Saline (PBS). One tube was treated with H$_2$O$_2$ to a final concentration of 2 mM, and a second tube received no treatment (control). Finally, after 20 min of treatment, 300 µl of each sample were used to measure fluorescence (GFP activity) employing a TECAN Infinite 200 PRO Nanoquant (excitation, 480 nm; emission, 509 nm) microplate reader. Emission values were normalized using the optical densities of treated and untreated strains; the measurement time for each was 3 min, for a total time of 45 min. Fluorescence and OD values were measured in triplicate. The specific fluorescence intensity was calculated using the methods of Eiamphungporn et al. [15], where the corresponding OD of the culture was used to normalize the initial and final fluorescence values of the construct and the empty vector. Specific fluorescence was calculated using the equation: $(\Delta$fluorescence/$\Delta$OD)$_{construct} - (\Delta$fluorescence/$\Delta$OD)$_{empty$vector} = \Delta$fluorescence/$\Delta$OD$_{empty$vector}$ for all strains subjected to treatments. All measurements were normalized using the values obtained from the wild-type strain grown under control conditions (no treatment).

RNA isolation and real-time quantitative PCR
Overnight bacterial cultures grown in LB were diluted (1:100). Cells were then grown to OD$_{600}$$\approx$0.4 and subjected to H$_2$O$_2$ treatment directly in the medium; one tube remained as an untreated control. After 20 min of incubation with H$_2$O$_2$, RNA was extracted using the acid–phenol method and the purified RNA was suspended in 30 µl of nuclease-free water. Finally, RNA integrity, quality, and quantity were verified using 1% agarose electrophoresis and A$_{260}$/A$_{280}$ ratio. Total RNA was treated with DNase I, and cDNA was synthesized using M-MLV RT (Promega) and random primers following the manufacturer’s instructions. cDNA was quantified by qRT-PCR using the primers shown in Additional file 2: Table S2. Relative quantification was performed using the Brilliant II SYBR Green qPCR Master Reagent Kit and the Mx3000P detection system (Stratagene); talB gene was used for normalization [5, 35]. Amplification efficiency was calculated using 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 the expression of the gene of interest.

Rifampicin assay
To evaluate the influence of the sRNA on ompX mRNA, we performed a rifampicin assay over time. Briefly, the strains (wild-type, ΔmicA, ΔcyaR, ΔoxyS and Δhfq) were grown in LB broth at 37°C with constant agitation. Once the cultures reached OD$_{600}$ ≈ 0.4 they were divided into two equal batches, one being the control whilst the other was treated with 2 mM H$_2$O$_2$. Then, at the predetermined time points (0, 5, 10, 15 and 20 min), 10 ml of culture were subjected to RNA extraction, cDNA preparation and the determination of ompX and talB expression by qRT-PCR, as described above. We performed two approaches: a rifampicin absent (–RIF) and rifampicin present (+RIF) assay. For the rifampicin present assay, we added rifampicin (20 µg/ml) when the cells originally reached an OD$_{600}$ ≈ 0.4.

Colony-forming units
To determine the colony-forming units (CFUs), S. Typhimurium 14028 s strains (wild-type, ΔmicA, ΔcyaR, ΔoxyS, ΔryhB and Δhfq) were grown to OD$_{600}$ ≈ 0.4 in LB using the corresponding antibiotic selection. Except in controls, H$_2$O$_2$ was added to a final concentration of 2 mM, and incubation took place with agitation at 37 °C for 20 min. After incubation, all strains were serially-diluted in PBS and spotted on LB agar. CFU were counted the following day, and the assay was replicated 5 times. We calculated percentage of survival using arbitrarily the wild-type strain grown under control conditions as 100% of survival.

Total intracellular ROS determination
To determine total intracellular ROS, the H$_2$DCFDA (Sigma-Aldrich) probe was used. Briefly, S. Typhimurium 14028 s strains (wild-type, ΔmicA, ΔcyaR, ΔoxyS, ΔryhB and Δhfq) were grown to OD$_{600}$ ≈ 0.4. Except in controls, H$_2$O$_2$ was added to a final concentration of 2 mM, and incubation took place with agitation at 37 °C for 20 min. After incubation, 300 µl of bacterial cultures were withdrawn in triplicate and placed in a 96-well microplate. Fluorescence was measured every 5 min, for a total of 45 min (excitation, 480 nm; emission, 520 nm). Total intracellular ROS were calculated
as: \((\Delta \text{fluorescence/time})/\Delta \text{OD}_{600}\). The final concentration of the probe was 10 µM in DMSO.

**In silico gene sequence evaluation**

To evaluate whether OxyS has binding sites on the ompX mRNA, we performed an in-silico analysis using the tool IntaRNA [4, 49] and the nucleotide sequences of the ompX mRNA and OxyS sRNA.

**Statistical analysis**

Gene-by-gene comparisons were performed as individual experiments for each time point using one-way ANOVAs with \(\alpha = 0.05\). Statistical analyses were performed with the Bonferroni correction comparing individual mutant strains with the wild-type strain. We used the Prism 7 software to perform all the data analysis.

**Results**

**H\(_2\)O\(_2\) stress increases levels of ompX mRNA but not of OmpX protein.**

Previously, our group reported that ompX transcript levels increased under peroxide stress [32]. To further evaluate the mechanism of ompX regulation under these conditions, we determined ompX transcript levels as well as promoter activity under H\(_2\)O\(_2\)-induced stress. Promoter activity was measured using the two putative promoters identified by Meccas et al. [30]. As shown previously, ompX transcript levels increased eight-times after exposure to hydrogen peroxide (Fig. 1A), while promoter activity increased almost three-fold (Fig. 1B).

To investigate whether the observed increase in ompX mRNA and promoter activity levels correlated with higher protein levels, we measured the OmpX protein by immunoblot. Unexpectedly, OmpX protein levels were not affected by the peroxide treatment (Fig. 1C). These results suggest that OmpX production may be subject to post-transcriptional regulation under these stress conditions. Previous evidence suggests that sRNAs such as MicA, RybB, CyaR and Hfq regulate this porin post-transcriptionally under standard growth conditions. For instance, CyaR contains a C-rich apical loop that sequesters the Shine-Dalgarno sequence of ompX mRNA and inhibits translation initiation [11, 17, 23, 34]. However, to our knowledge, the specific mechanism that occurs under ROS-enriched conditions has not been elucidated and should take into consideration the role of molecules that are targeted by hydrogen peroxide and that can potentially lose function as a consequence of oxidative damage.

**ompX is regulated at the post-transcriptional level in response to H\(_2\)O\(_2\)**

In *Escherichia coli*, ompX mRNA is regulated at the post-transcriptional level by CyaR and MicA [22]. In silico analysis revealed the presence of a putative interaction domain between OxyS and ompX mRNA (Fig. 2), suggesting that OxyS could participate in some way in the regulation of ompX mRNA under oxidative stress.

Given their previously described roles in model bacteria such as *Escherichia coli*, we determined whether CyaR, MicA, or OxyS play relevant roles during peroxide stress in S. Typhimurium by examining bacterial survival and intracellular ROS accumulation. Bacterial strains with the deletion of each sRNA show decreased survival in comparison to the wild-type control, especially after deletion of micA (Fig. 3A). Furthermore, intracellular ROS accumulation is also greater in mutant strains (Fig. 3B), suggesting that this stressor has an even more detrimental effect on the overall survival of the bacteria when the function of these sRNAs is absent.

Moreover, the amount of ompX transcript significantly increases in the wild-type strain after ROS-induced stress, but not in the isogenic mutant strains for each sRNA (Fig. 3C), suggesting a role in the regulation of the porin during ROS-resistance. The results suggest that CyaR, MicA, and OxyS are required to maintain ompX levels under oxidative stress 20 min post-treatment, indicating that at least partially, this mechanism of survival to ROS-induced stress is MicA-, CyaR- and OxyS-dependent.

To evaluate the post-transcriptional regulation of the ompX mRNA in response to peroxide stress, we assessed ompX expression after treating bacteria with rifampicin during 20 min and measured expression at 5 min intervals. Rifampicin is an antibiotic that affects the RNA polymerase, specifically the elongation process; therefore, changes in mRNA content after inhibiting mRNA production are due to post-transcriptional processes [48]. This approach enabled us to generate more evidence regarding the post-transcriptional regulation of OmpX under ROS-related stress, by using an antibiotic that inhibits RNA polymerase and observing changes in mRNA production associated with post-transcriptional processes. Also, as controls we measured the effect of rifampicin on the wild-type strain and found that there is a significant decrease in the stability and amount of ompX expression (Additional file 3: Fig. S1). Moreover, we found that adding 5 µg/mL rifampicin reduced the levels of transcripts (Additional file 4: Fig. S2), validating the use of this approach to measure ompX expression under ROS stress. The results indicate that there is no difference regarding stability and amount of ompX in the control conditions with (Fig. 4A) or without (Fig. 4B) the antibiotic treatment. However, once bacteria were
under ROS-induced stress, we found a statistically significant decrease in the amount of *ompX* transcripts in all mutant strains compared to the wild-type strain (Fig. 4C). Using the rifampicin treatment, we determined that the decreased amount of transcript of the mutant strains compared to the wild-type is a consequence of the activity of the sRNA during post-transcriptional regulation, as the levels detected in the wild-type strains are significantly

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**Fig. 1** Analysis of *ompX* expression. **A** mRNA level of *ompX* in the wild-type strain of *S. Typhimurium* 14028. **B** Fluorescence activity of GFP under the control of the *ompX* promoter (-353 to -1) in the wild-type *S. Typhimurium* background. **C** Immunodetection of the OmpX:3xFlag protein, measured after exposure of the strain to 2 mM H$_2$O$_2$ for 20, 45 and 60 min. The control received no treatment. Ten µg of total proteins were loaded. White bars represent the control (no treatment), and red bars represent cells treated with 2 mM H$_2$O$_2$. The graph represents the average of 3 independent experiments (mean ± SD or SE)
higher than those in the strains that lack the genes for the different sRNA tested.

These findings indicate that MicA, CyaR, OxyS and Hfq exert their regulatory functions once the ROS-induced stress is signaled to the cell inducing the expression of this small porin. Our results allow us to suggest that these three sRNAs (MicA, CyaR and OxyS) are required to stabilize the \textit{ompX} mRNA in an Hfq-dependent manner, when Salmonella faces hydrogen peroxide-induced stress.

\textbf{Discussion}

Previous studies showed that increased OmpX expression alters the levels of other outer membrane porins, such as OmpC and OmpF, and increases sigma E (\(\sigma^E\))-containing RNA polymerase activity [30]. Our results show that under \(H_2O_2\)-induced stress, the levels of \(ompX\) transcript increased as well as its promoter activity (Fig. 1A, B), yet there was no increase in OmpX protein levels (Fig. 1C), suggesting that \(ompX\) is regulated post-transcriptionally. We found that \(ompX\) mRNA is regulated by MicA, OxyS, and CyaR in response to \(H_2O_2\). Furthermore, we speculate that lower \(ompX\) mRNA levels, observed in response to ROS, result from inhibition in translation, given that mRNA stability decreases when translation initiation is halted [8, 13].

Supporting this view, MicA and OxyS affected OmpX protein levels (Additional file 5: Fig. S3). MicA expression, induced by various stresses (some of which can be found inside the phagosome), is regulated by \(\sigma^E\), which plays a key role in the oxidative stress response [19, 20,
inhibiting the expression of ompW and phoP [46]. Srikumar et al. [44] demonstrated that MicA and OxyS are upregulated in Salmonella during infection of murine macrophages. The former (MicA) can adopt various conformations, and dimerization inhibits its function [46]. OxyS regulates rpoS, encoding an alternative
sigma factor required for responses to low temperatures, osmotic shock, and membrane stress [38] as well as fhlA, encoding a protein involved in metabolic stress adaptation [2]. cyaR expression is slightly induced in Yersenia pestis exposed to peroxide stress [50] and is regulated under nutrient deprivation, a condition that can be found in Salmonella-containing vacuoles (SCV; [11, 17, 20]). All this data supports our hypothesis that these sRNAs participate in ROS response and could function to modulate positively ompX transcripts.

Previous studies suggested that protein production depends on three factors: transcription rate, mRNA degradation and mRNA concentration, factors that depend on the cell status [12]. When mRNA levels increase as a function of increased transcription, mRNA stability is strongly affected. Thus, mRNA stability depends on mRNA concentration [33], among other factors. In the same study, the authors suggest that mRNA levels must reach an equilibrium imposed by the condition and energetic status of the cell, a way to control and reduce the energetic cost associated with the production of new proteins [33].

OmpX is essential for invasion of various bacterial species such as Enterobacter, Yersinia, and Cronobacter [9, 24, 25], and for iron homeostasis in E. coli [28]. Therefore, fine regulation of OmpX under oxidative stress could be an adaptation to the stress encountered inside macrophages, where the bacteria attempt to balance the influx of essential metabolites with the uptake of ROS through this porin. Recently, it has been found that the OmpX porin is involved in biofilm formation and curli production [26]. Thus, these critical roles require fine regulation in response to specific conditions that enable bacteria to adapt efficiently. Further studies are necessary to determine the specific role of this protein during the oxidative stress, including other strategies such as antisense RNA, proteomic and in silico analyses of the structural relationships among all molecules.
Conclusions

Our results show that ompX mRNA is regulated in response to H$_2$O$_2$ by the sRNAs CyaR, MicA and OxyS. These data represent a step forward in this area and provide additional insights into the complex regulation of ompX in response to H$_2$O$_2$-induced stress, a physiologically relevant condition encountered by S. Typhimurium during its infection cycle.

Abbreviations

ompX: Outer membrane porin X; mRNA: Messenger Ribonucleic Acid; sRNA: Small Ribonucleic Acid; S. Typhimurium; Salmonella enterica Serovar Typhimurium; O$_2^\cdot-$: Superoxide anion; H$_2$O$_2$: Hydrogen peroxide; ROS: Reactive Oxygen Species; RIF: Rifampicin; LB: Lennox Broth; OD: Optical density; CFU: Colony forming Units; GFP: Green Fluorescence Protein.

Supplementary Information

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Authors' contributions

CPS and EHM conceived and designed the study. ACB, DL, AC, CEC, GIK, MSB, CR, ME carried out all experiments and procedures. CPS, JCS, CPE contributed to analyses and the writing of the manuscript. CPS, RF, AAH provided materials, critical advice, and analyses. All authors read and approved the final manuscript.

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Availability of data and materials

All data is available upon request.

Declarations

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Does not apply.

CONSENT FOR PUBLICATION

All authors have consent for the publication of this manuscript.

COMPETING INTERESTS

The authors declare no competing interest.

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