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The small RNA RyhB homologs from *Salmonella typhimurium* participate in the response to *S*-nitrosoglutathione-induced stress

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ABSTRACT

Typically, the expression of sRNAs is activated in response to environmental stimuli in order to regulate gene expression through post-transcriptional mechanisms. In the present work we show that the *Salmonella typhimurium* paralogous sRNAs RyhB-1 and RyhB-2 are induced in response to the nitrosating agent *S*-nitrosoglutathione (GSNO). Inactivation of these sRNAs decreased *S. typhimurium* resistance to GSNO and increased the levels of nitrosylated proteins. These results prompted us to evaluate a possible role of these sRNAs in nitrosative stress resistance. RNA profiling was used as a screen to identify novel RyhB-1 and RyhB-2 regulated targets. A subset of genes was filtered based on their potential role in the response to nitrosative stress and their expression was analyzed by quantitative RT-PCR in wild type, single and double mutant strains (Δ*ryhB1*, Δ*ryhB2* and Δ*ryhB1* Δ*ryhB2*) treated with GSNO. In response to GSNO RyhB-1 and RyhB-2 negatively regulate the expression of the genes *cyoABC* (cytochrome *bo* oxidase), *cydB* (cytochrome *bd* oxidase), *cybC* (cytochrome b-562), and positively regulate the *nirBCD* operon (nitrite reductase system). Together, these results suggest that RyhB-1 and RyhB-2 finely tune the expression of genes coding for cytochrome oxidases and the nitrate reductase system, allowing the cell to cope with GSNO-induced stress.

1. Introduction

The ability of bacteria to survive and replicate within host cells is crucial for its success as a pathogen. During infection of the host, a cascade of anti-bacterial processes are initiated by professional phagocytes, including the production of nitric oxide (NO) via the inducible NO synthase [1]. In turn, NO can be converted to other reactive nitrogen species (RNS) such as nitrogen dioxide (NO_2), peroxynitrite ($ONOO^-$) and dinitrogen trioxide (N_2O_3) [2], and also into thiol-containing molecules such as *S*-nitrosoglutathione (GSNO) [3]. Nitric oxide and nitrosating agents like GSNO exert powerful antimicrobial effects by reacting with molecular targets such as transition metals, thiols, lipids, and DNA [2]. *Salmonella enterica* and many other enteric pathogens possess a number of enzymes that metabolize NO and other RNS to non-toxic metabolites; these enzymes include flavohemoglobin (Hmp), flavorubredoxin and associated oxidoreductase (NorVW), and cytochrome c nitrite reductase (NrfA) [4–8]. For bacterial pathogens like *Salmonella*, effective detoxification systems for antimicrobial reactive oxygen and nitrogen species are important to establish a successful infection and to survive inside the host. In this context, regulatory small RNAs play a critical role in the adaptation to fast changing environments by regulating gene expression at the post-transcriptional level via base-pairing with target mRNAs. The expression of many *Salmonella typhimurium* sRNAs has been directly monitored inside macrophages or in media mimicking infection-relevant stress conditions, i.e., high osmolarity, extreme pH, nutrients starvation, oxygen limitation, oxidative stress and conditions of iron and magnesium limitation [9–13].

Abbreviations: GSNO, *S*-nitrosoglutathione; RNS, reactive nitrogen species.

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The goal of the present work was to determine, for the first time, if sRNAs play a role of in the response to nitrosative stress. For this purpose, we used the model *S. typhimurium* and the nitrosating agent GSNO, a NO donor compound commonly used to study nitrosative stress [14]. Out of eight sRNAs that were tested, only RyhB-1 and its island-encoded paralog RyhB-2 (also called IsrE) were induced in response to GSNO. These two *S. typhimurium* paralogous sRNAs are well characterized iron responsive regulators and members of the Fur (Ferric Uptake regulator) regulon. RyhB-1 and RyhB-2 regulate the expression of non-essential iron-containing proteins in response to iron depletion (e.g., *acnA*, *acnB*, *ftn* and *sodB*) and of genes implicated in motility for the case of RyhB-2 (*flgJ*, *cheY* and *fliF*) [11,15–17]. To better understand the role of RyhB-1 and RyhB-2 in the response to GSNO, the expression of novel candidate targets related with the response to nitrosative stress was tested. Our results support a regulatory role for RyhB-1 and RyhB-2 in the bacterial response to nitrosative stress.

2. Material and methods

2.1. Bacterial strains and growth conditions

The strains and plasmids used in this study are listed in Supplementary Table S1. Bacteria were grown routinely at 37 °C in Luria-Bertani medium (LB) and aerated by shaking (250 rpm). When required, LB was supplemented with ampicillin (100 mg l^{-1}), chloramphenicol (25 mg l^{-1}) or kanamycin (50 mg l^{-1}). For growth curves, bacteria were grown overnight in 5 ml of LB medium at 37 °C with shaking. 1:1000 dilutions of the overnight cultures were grown in 25 ml of LB. The cells were treated at an OD_{600} of 0.3 with 2 mM GSNO. Aliquots were withdrawn at different time points after GSNO treatment and the OD_{600} was recorded.

2.2. S-Nitrosylated protein detection assay

The *S*-nitrosylation of cellular proteins was determined using the "S-Nitrosylated Protein Detection Assay Kit" (Cayman®). This assay employs a modification of the "Biotin-switch" method [18–19] to allow for the direct visualization of *S*-nitrosylated proteins in whole cells by western blot analysis. Using this method, free SH groups are first blocked and any S–NO bonds present in the sample are then cleaved. Biotinylation of the newly formed SH groups provides the basis for the colorimetric visualization using streptavidin. Briefly, *S. typhimurium* cells grown in LB medium to an OD_{600} of 0.3 were treated with 2 mM GSNO for 20 min and *S*-nitrosylated proteins were determined following the manufacturer's instructions for cell suspension cultures.

2.3. RNA extraction, reverse transcription and Real-time PCR (qRT-PCR)

Total RNA extraction and qRT-PCR analyses were performed as previously described [20]. Briefly, RNA was obtained with the acid-phenol method from cells grown in LB, treated in exponential phase (OD_{600} of 0.4) with 2 mM GSNO for 20 min. Specific primers used are listed in Supplementary Table S2.

2.4. Microarray analysis

All experimental procedures, data acquisition and normalization were performed as previously described [21]. RyhB-2 was expressed from a plasmid under the control of the inducible TAC promoter (pIDT-RyhB2). Briefly, cells carrying pIDT-RyhB2 (14028s-pRyhB2) were grown in LB to an OD_{600} of 0.4 and treated with 1 mM IPTG for 20 min. Experiments were performed in duplicate on different days. After induction, mRNA was extracted, cDNA was generated, and hybridized to a ~387.000 50-mer NimbleGen microarray (Roche NimbleGen). After data acquisition, intensity values from this experiment and from our recent study with strain 14028s grown under the same conditions (GEO ID: GSE34134; [21]) were background subtracted, and normalized within (median) and between (quantile) the arrays using WebarrayDB [22]. After data normalization, *T*-test was performed using the software MeV TM4 [23] to determine uncorrected p-values. False Discovery Rate adjusted q values were calculated using QVALUE in Bioconductor [24]. For the purposes of this study, genes with a q value $\leqslant 0.05$ and changes in gene expression of at least 1.5-fold (14028s-pRyhB2/14028s, Supplementary Table S3) were considered potentially significant. To validate the results from the microarray analysis (Table 1), strains 14028s-pRyhB2 and 14028s-pIDT (cells carrying the control plasmid) were grown and treated with IPTG as described above, and the transcript levels of selected genes was measured by qRT-PCR. The microarray data has been deposited in GEO ID: GSE58110 (http://www.ncbi.nlm.nih. gov/geo/).

3. Results

3.1. The sRNAs RyhB-1 and RyhB-2 are induced under nitrosative stress conditions

To identify *S. typhimurium* sRNAs regulated in response to GSNO, we tested the expression of eight sRNAs previously detected under stress conditions that *Salmonella* encounters upon infection of the host [11,25]. Bacteria were grown until mid-log phase and exposed to GSNO, and expression was measured by qRT-PCR. Of the eight sRNAs that were tested, only RyhB-1 and RyhB-2 were up-regulated in response to GSNO, with an increase of 8- and 5-times ($p < 0.005$), respectively, as compared to untreated cells (Fig. 1).

3.2. RyhB-1 and RyhB-2 are required for GSNO resistance

To determine if RyhB-1 and/or RyhB-2 are required for nitrosative stress resistance, growth curves were generated for *S. typhimurium* strains exposed to GSNO. The nitrosating agent delayed growth of strains Δ*ryhB1*, Δ*ryhB2* and Δ*ryhB1* Δ*ryhB2*, as compared to strain 14028s (wild type), with the Δ*ryhB1* Δ*ryhB2* mutant showing a less pronounced growth defect than the individual mutant strains (Fig 2A). By using a biotin-switch method, the levels of *S*-nitrosylated proteins were determined. In a similar pattern as for growth curve experiments, the levels of *S*-nitrosylated proteins were higher in strains Δ*ryhB1* and Δ*ryhB2*, while in the double mutant the levels were slightly increased with respect to strain 14028s (Fig 2B). The fact that the double mutant strain did not exhibit the same phenotype of the single mutant strains or a synergistic defect suggests that the roles of RyhB-1 and RyhB-2 are not absolutely redundant, as previously observed under oxidative stress [20]. Taken together, these results support a role for both RyhBs in the response to nitrosative stress induced by GSNO.

3.3. RyhB-2 overexpression affects the levels of cytochrome oxidase complexes and of the nitrite reductase system, and reveals potential targets under GSNO-induced stress

Since island encoded sRNAs play an important role in regulating bacterial adaptation to stress conditions, and thus control virulence [11], we firstly focused our study on the island-encoded sRNA RyhB-2. As a preliminary approach to identify genes regulated in a RyhB-dependent manner and that could be related with nitrosative stress resistance, the expression profile of a strain

Table 1
Microarray analysis and validation by qRT-PCR.

Gene	Gene product	Microarray	qRT-PCR
		Fold change[a]	Fold change[b]
cyoA	Cytochrome *bo* ubiquinol oxidase subunit II	−1.67	−2.66
cyoB	Cytochrome *bo* ubiquinol oxidase subunit I	−1.53	−2.79
cyoC	Cytochrome *bo* ubiquinol oxidase subunit III	−1.64	−2.21
cybC	Cytochrome b562	−2.71	−3.50
cydB	Cytochrome *bd* ubiquinol oxidase subunit II	−1.77	−1.91
nirB	Nitrite reductase large subunit	5.26	4.43
nirD	Nitrite reductase small subunit	4.42	3.13
nirC	Nitrite transporter	2.79	3.20

[a] Genes with a fold change (14028s-pRyhB2/14028s) of $\geqslant 1.5$ and an FDR q value of $\leqslant 0.05$ were considered to be differentially expressed. Here, the expression profile of the strain 14028s-pRyhB2 was compared with that of the strain 14028s from our previous study [21].

[b] Values are based on fold change (14028s-pRyhB2/14028s-pIDT) calculated from ΔΔCt values and were $\log_2$ transformed.

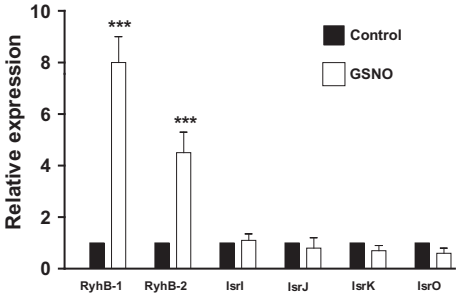

Fig. 1. Expression of *S. typhimurium* sRNAs in response to GSNO-induced stress. The wild type strain was grown aerobically in LB to $OD_{600} \sim 0.3$ and then treated with 2 mM GSNO for 20 min to analyze the expression of selected sRNAs (indicated in figure) by qRT-PCR. Control cells received no treatment. Asterisks represent statistical significant differences between control and treated cells (***$p < 0.005$). Data represent the means ± standard deviations ($n = 3$).

overexpressing RyhB-2 (14028s-pRyhB2) was compared with that of strain 14028s from our previous study [21]. The analysis showed that RyhB-2 overexpression (5.5-fold increase vs. strain 14028s; Supplementary Table S3) increased the mRNA levels of the *nirBCD* operon (nitrite reductase system), and decreased the levels of *cyoABC* (cytochrome *bo* oxidase), *cydB* (cytochrome *bd* oxidase) and *cybC* (cytochrome b-562), among others (Table 1, Supplementary Table S3). This was confirmed by qRT-PCR using strains 14028s-pRyhB2 and 14028s-pIDT (Table 1), indicating that the result from the microarray analysis is due to RyhB-2 overexpression. Interestingly, the transcript levels of the same genes also changed when strain 14028s was exposed to GSNO (Fig. 3A). Exposure to the toxic compound increased the expression of the *nirBCD* operon (Fig. 3A), as observed with RyhB-2 overexpression (Table 1), suggesting that RyhB-2 could be implicated in the positive regulation. In the case of the genes *cyoABC*, *cydB* and *cybC*, GSNO treatment induced their expression in strain 14028s (Fig. 3A), the opposite to what was observed with RyhB-2 overexpression (Table 1). This suggests that if RyhB-2 negatively regulates these genes in response to GSNO, the effect is only partial.

3.4. RyhB-1 and RyhB-2 regulate the expression of cytochrome oxidase complexes and of the nitrite reductase system under GSNO-induced stress

To directly test if RyhB-1 and RyhB-2 regulate the expression of the *nirBCD* operon and cytochrome genes in response to GSNO, their expression was monitored in strains Δ*ryhB1*, Δ*ryhB2* and Δ*ryhB1* Δ*ryhB2* treated with the toxic compound. The transcript levels of *cyoA*, *cyoB*, *cyoC*, *cydB* and *cybC* significantly increased in all three mutant backgrounds, although to different extents, as compared to strain 14028s treated with GSNO (Fig. 3B). This indicates that even when *cyoA*, *cyoB*, *cyoC*, *cydB* and *cybC* were up-regulated in all the tested strains, expression was significantly higher in the strains lacking the sRNAs, and hence both RyhB-1 and RyhB-2 negatively affect their expression. In the case of *nirB*, *nirD* and *nirC*, the transcript levels significantly decreased after GSNO treatment in the single and double mutant strains when compared to strain 14028s under the same condition (Fig. 3B). This result is in agreement with the sRNA overexpression assay (Table 1), and indicates that RyhB-1 and RyhB-2 positively regulate their expression (Fig. 3B). Complementation of the single mutant strains confirmed that the changes in gene expression were due to deletion of the *ryhB* genes (Supplementary Fig. S1).

4. Discussion

The sRNAs RyhB-1 and RyhB-2 have been previously characterized for their role in iron homeostasis [11,15–17,26], however, we recently demonstrated that they also participate in the response to hydrogen peroxide [20]. The fact that these sRNAs are also induced

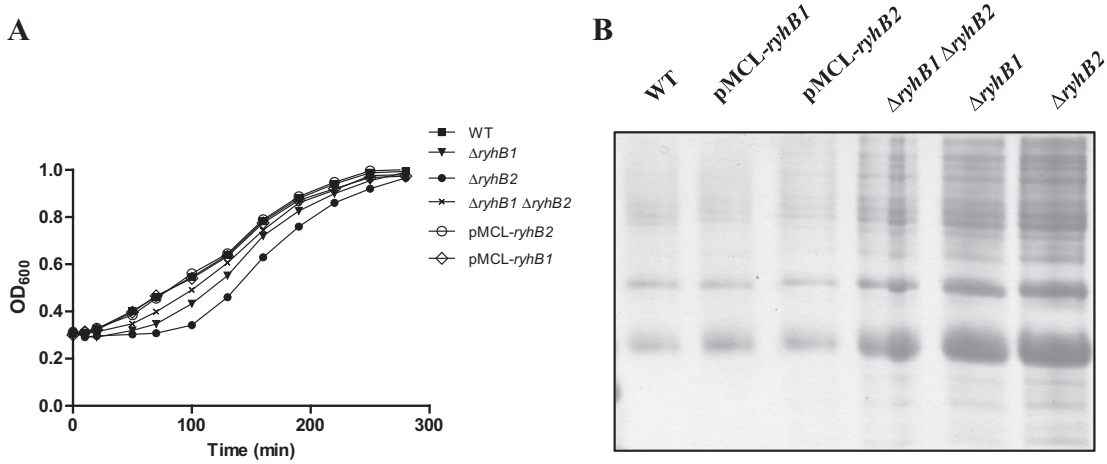

Fig. 2. Growth curves and *S*-nitrosylated protein profiles of *S. typhimurium ryhB* mutants exposed to GSNO. (A) For growth curves, wild type (WT), Δ*ryhB1*, Δ*ryhB2* and Δ*ryhB1* Δ*ryhB2* strains were grown aerobically in LB media until an $OD_{600} \sim 0.3$ and treated with 2 mM GSNO. (B) *S*-Nitrosylation of total proteins from each strain was determined under the GSNO-stressed condition described above and each lane contains 20 μg of total proteins. The experiments include the corresponding complemented strains pMCL-*ryhB1* and pMCL-*ryhB2*. Results are representative of at least three independent assays.

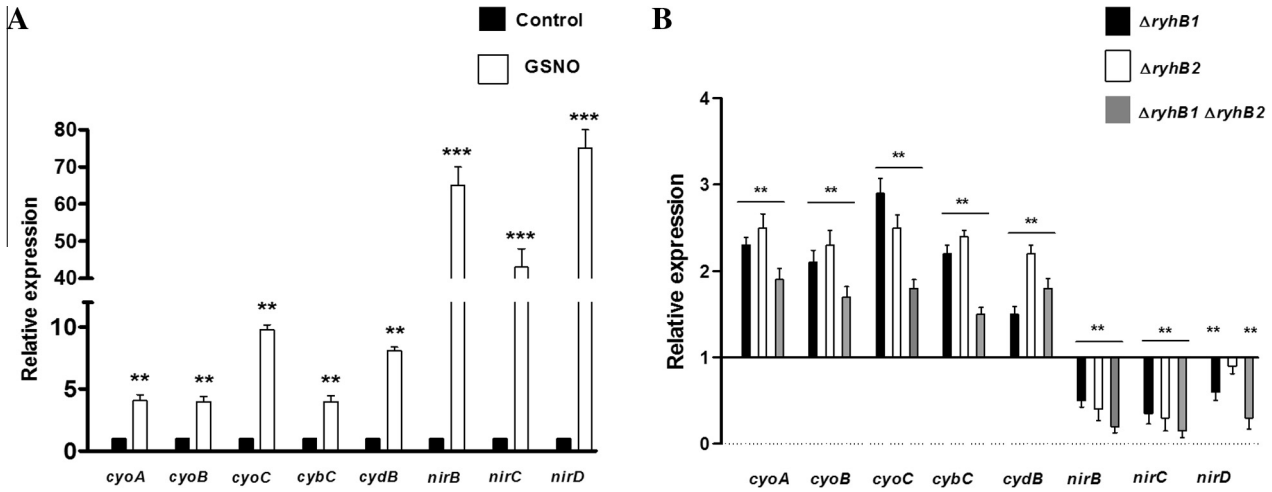

Fig. 3. Validation of RyhB-1 and RyhB-2 targets under GSNO-induced stress. Total RNA was extracted from wild type, Δ*ryhB1*, Δ*ryhB2* and Δ*ryhB1* Δ*ryhB-2* strains grown aerobically in LB media until an $OD_{600} \sim 0.3$ and treated with 2 mM GSNO for 20 min to analyze the expression by qRT-PCR. All values were normalized to the levels of the 16s rRNA. (A) The results represent the expression in the wild type strain treated with GSNO relative to that of wild type control (untreated). (B) The results represent the expression in the mutant strains treated with GSNO relative to that of wild type strain treated with GSNO. Asterisks represent statistical significant differences between wild type and mutant strains (**$p < 0.05$; *** $p < 0.001$). Data represent the means ± standard deviations ($n = 3$).

under nitrosative stress (Fig.1) and that they regulate the expression of genes related with this condition (Table 1 and Fig. 3A and B) suggests that their functions are not only limited to iron homeostasis. Seemingly these sRNAs possess the ability to integrate a global response to multiple stresses encountered inside macrophages, i.e., iron starvation, oxidative and nitrosative stress. Supporting this view, the expression of RyhB-1 and RyhB-2 is significantly induced when *S. typhimurium* resides in macrophages [11] and their orthologs from *S. typhi*, RfrA and RfrB, are required for optimal intracellular replication in macrophages [27].

In the present work we used GSNO as an agent of nitrosative stress because NO is released from this compound by homolytic decomposition. NO is highly reactive with metal centers, including Fe–S clusters or hemes of metabolic enzymes to form iron-nitrosyl adducts [28–31]. Even though the heme of cytochrome oxidases is one of the preferred targets of NO cytotoxicity [32–33], both the cell content of heme and the respiratory cytochrome oxidases *bd* are required for a proper response to nitrosative stress, further contributing to the virulence of many bacterial pathogens [34–39]. Studies on NO reactivity with the catalytic intermediates of cytochrome *bd* led to the discovery that the enzyme, though having a higher affinity for NO than does cytochrome *bo*, exhibits an unusually fast NO dissociation rate from the active site, unlike most heme-containing proteins. This allows the NO-inhibited cytochrome *bd* to quickly recover its activity, reducing its sensitivity to NO and protecting the proton-translocating and O_2-reducing capacities of cytochrome *bo* [35]. Thus, these heme-containing enzymes contribute to counteract NO effects, but also their heme groups have the potential to cause toxicity at high concentrations [40–41]. This heme paradox requires that the intracellular pool remains tightly regulated under specific stress conditions. In this context, we propose that the sRNAs RyhB-1 and RyhB-2 could aid bacteria to respond to stress by modulating heme levels through the down-regulation of cytochromes genes, thus limiting the demand for heme and its cytotoxic effects associated with the iron released from them. This could explain why the expression of cytochrome genes is increased in a controlled manner in wild type cells treated with GSNO (Fig. 3A and B), but not in the Δ*ryhB*1, Δ*ryhB2* and Δ*ryhB1* Δ*ryhB2* mutant strains, where expression is even higher, which could lead to increased GSNO susceptibility (Fig. 2A and B). Supporting these observations Li *et al.*, [42] recently reported that RyhB constitutive expression down-regulates heme biosynthesis and cytochrome *bd* expression in *Escherichia coli.*

On the other hand, up-regulation of the *nir* operon in strain 14028s after GSNO exposure (Fig. 3A) can be directly related with the ability of the bacteria to detoxify NO. When bacteria are inside the host, specifically in the *Salmonella*-containing vacuole, NO can be auto-oxidized to nitrite [43] and then incorporated into the bacteria. Bacterial nitrite accumulation is critical because it can be reduced to NO as a side reaction of nitrate reductase (NarGHI) [44], so that cytoplasmic nitrite is immediately removed by a nitrite reductase yielding ammonium [45]. This is performed mainly by the NADH-dependent siroheme-containing nitrite reductase (NirBD), which consequently depends on the nitrite transporter NirC [46]. NirC is directly involved in nitrosative stress and the virulence of *S. typhimurium*. In fact, NirC inactivation decreases the NO-dependent *Salmonella* intracellular proliferation in activated macrophages [47]. Here we report that all three genes of this system, namely *nirB*, *nirC* and *nirD*, were induced in GSNO-treated cells in a RyhB-dependent manner, supporting the participation of these sRNAs in the response to nitrosative stress.

Although we cannot rule out the possibility that some of the genes analyzed here are indirectly regulated by RyhB-1 and RyhB-2, the results presented herein argue in favor of an integrative role for these sRNAs in the response to multiple stresses.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2014.06.031.

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