Microbiology Papers in Press. Published February 7, 2013 as doi:10.1099/mic.0.066050-0

ompW is cooperatively up-regulated by MarA and SoxS in response to menadione.

Collao B.[1†], Morales E.H.[1†], Gil F.[1], Calderón I.L.[1], Saavedra C.P.[1*]

[1]Laboratorio de Microbiología Molecular, Facultad de Ciencias Biológicas, Universidad Andres Bello, Santiago, Chile.

*Corresponding author. Claudia Saavedra, Laboratorio de Microbiología Molecular, Facultad de Ciencias Biológicas, Universidad Andres Bello, República 217, Santiago, Chile. Telephone: 56-2-6618664 Fax: 56-2-6618390 E-mail: csaavedra@unab.cl

† Both authors contributed equally

Keywords: marA/rob/soxS regulon, oxidative stress, regulation transcriptional.

Abstract

OmpW is a minor porin whose biological function has not been clearly defined. Evidence obtained in our laboratory indicates that in *S*. Typhimurium, its expression is activated by SoxS upon exposure to paraquat and is required for its resistance. SoxS belongs to the AraC family of transcriptional regulators, as well as MarA and Rob. Due to their high structural similarity, the genes under their control have been grouped in the *mar/sox/rob* regulon, which present a DNA binding consensus sequence denominated the *marsox* box. In this work, we evaluated the role of the transcription factors MarA, SoxS and Rob of *S*. Typhimurium in regulating *ompW* expression in response to menadione. We determined the transcript and protein levels of OmpW in different genetic backgrounds; in the wild type and Δ*rob* strains, *ompW* was up-regulated in response to menadione, while in the Δ*marA* and Δ*soxS* strains the induction was abolished. In a double *marA soxS* mutant, *ompW* transcript levels were lowered after exposure to menadione, and only complementing in *trans* with both genes restored the positive regulation. Using transcriptional fusions and EMSAs with mutant versions of the promoter region we demonstrated that two of the predicted sites were functional. Additionally, we demonstrate that MarA increases the affinity of SoxS for the *ompW* promoter region. In conclusion, our study shows that *ompW* is up-regulated in response to menadione in a cooperative manner by MarA and SoxS through a direct interaction with the promoter region.

Introduction

Porins are aqueous channels that allow the passive diffusion of hydrophilic solutes, nutrients or toxic compounds through the bacterial outer membrane and participate, at least in part, in the ability of bacteria to adapt to diverse environments, in drug resistance mechanisms and in bacterial pathogenesis (Benz & Bauer, 1988; Chatfield *et al*., 1991; Jeanteur *et al*., 1991; Weiss *et al*., 1991; Groisman & Ochman, 1994; Nikaido, 1996; Zgurskaya & Nikaido, 2000; Koebnik *et al*., 2000; Rodríguez-Morales *et al*., 2006). Some years ago, Morimyo (1988) isolated and characterized *Escherichia coli* (*E. coli*) mutants sensitive to paraquat (PQ), a superoxide-generating compound (Hassan & Fridovich, 1979).The deleted region in *E. coli* is highly conserved in *Salmonella enterica* serovar Typhimurium (*S.* Typhimurium) and contains the *ompW* gene (Gil et al., 2007), which encodes for a minor porin that has been well studied and its structure has been described in *E. coli* and *Vibrio cholerae*, although its biological function has not been clearly defined (Nandi *et al*., 2005; Hong *et al*., 2006). It is thought to be involved in osmoregulation, since in *V. alginolyticus* high salt concentrations (NaCl 4%) induce its expression (Xu *et al*., 2005). Furthermore, a *S.* Typhimurium ceftriaxone-resistant strain showed decreased expression of *ompW*, suggesting that it might be involved in the uptake of this antibiotic (Hu *et al*., 2005). Evidence obtained in our laboratory indicates that in *S*. Typhimurium 14028s OmpW expression is increased in the presence of paraquat and mediates its resistance (Gil *et al*., 2007).

The cellular response to superoxide (O_2^-) is regulated at the transcriptional level by the SoxRS system (Greenberg *et al*., 1990). Upon exposure to O_2^- and/or ammonium quaternary compounds, SoxR is oxidized and converted to an active form that induces the

65 transcription of *soxS*, which binds to the promoter regions of several genes whose products
66 are involved in response to oxidative damage (Storz & Imlay, 1999; Scandalios, 2002;
67 Imlay, 2008; Gu & Imlay, 2011). In this context, the evidence supports a model in which
68 ammonium quaternary compounds are responsible for SoxR activation (Krapp *et al*., 2011;
69 Gu & Imlay, 2011), however, it has been recently confirmed that O_2^- is also able to oxidize
70 its [2Fe-2S] cluster (Fujikawa *et al*., 2012). SoxS belongs to the AraC family of
71 transcriptional regulators, of which MarA and Rob are also members (Martin & Rosner,
72 2002). In *E. coli* MarA, SoxS and the N-terminal domain of Rob, which include the DNA
73 binding domain, share approximately 50% of amino acid identity (Jair *et al*., 1995; Jair *et*
74 *al*., 1996; Tobes & Ramos, 2002). The *mar/sox/rob* regulons overlap and together they co-
75 regulate, by direct binding to the promoter regions, more than 40 different genes (Aono *et*
76 *al*., 1998; Martin *et al*., 1999; Martin *et al*,. 2000; Martin & Rosner, 2002). Expression of
77 MarA, SoxS and Rob is increased upon exposure to a wide variety of signals. MarA is
78 increased in response to weak acid conditions and salicylate treatment (Pomposiello *et al*.,
79 2001); SoxS in response to nitric oxide, superoxide and ammonium quaternary compounds
80 (Li & Demple, 1994; Vasil'eva *et al*., 2001) and Rob after treatment with bile salts and
81 dipyridyl (Storz & Imlay 1999; Semchyshyn *et al*., 2005; Kwon *et al*., 2000). In *E. coli*,
82 their up-regulation is correlated with changes in the expression of genes involved in the
83 efflux of antibiotics (*acrAB*, *tolC*), decrease in outer membrane permeability (*micF*),
84 superoxide resistance (*fpr*, *sodA*), DNA repair systems (*nfo*) and those with unknown
85 function (Aono *et al*., 1998; Pomposiello *et al*., 2001; Chollet *et al*., 2002; Chollet *et al*.,
86 2004; Giro *et al*., 2006; Lee *et al*., 2009). Due to the high structural similarity among these
87 proteins, genes under their control have been denominated the *mar/sox/rob* regulon which
88 present a DNA binding consensus sequence at their promoter regions, denominated the

marsox box which is degenerate,,asymmetric (AYnGCACnnWnnRYYAAAY) and has been determined at various locations on the chromosome of *E. coli* (Martin *et al*., 2008; Aono *et al*., 1998; Martin *et al*., 1999; Martin *et al*., 2000; Martin & Rosner, 2002). These binding sites are also configured in a specific orientation and relative distance to the upstream -35 and -10 elements, to which RNA polymerase binds (Martin & Rosner, 2002; Selke *et al*., 2007).

Previous work in our laboratory determined that *ompW* is regulated by SoxS in response to PQ, and a *marsox* box has been defined at its promoter region with the sequence 5' TTTGCATAGCGTGAATATGTCAAAATTGAT 3' (Gil *et al*., 2009). Since the binding sites of the members of the *mar/sox/rob* regulon are similar, in *S*. Typhimurium Rob and MarA might also regulate *ompW* in response to menadione, another superoxide-generating compound (Kato *et al*., 1994).

In the present work, we evaluated the effect of menadione in *ompW* expression and the role of MarA and SoxS in the response. To evaluate the changes after exposure to menadione, we determined the transcript and protein levels of OmpW in the different genetic backgrounds after exposure to the toxic compound. In the wild type and Δ*rob* strains, *ompW* was up-regulated in response to menadione, while deletion of MarA or SoxS abolished the regulation. Bioinformatic analyses predicted the presence of three potential *marsox* boxes at the *ompW* promoter region, including the one previously described by Gil *et al*., (2009). Using transcriptional fusions and electrophoretic mobility shift assays (EMSAs) with the wild type and mutated promoter region we demonstrated that two of the predicted sites were functional. Interestingly, in a double *marA soxS* mutant strain *ompW* transcript levels were lowered after menadione exposure, and only complementing in *trans* with both genes was able to restore the positive regulation observed in the wild type strain.

In conclusion, we demonstrate that in response to menadione, MarA and SoxS cooperatively regulate *ompW* through a direct interaction with the promoter region.

Methods

Bacterial strains and growth conditions

Salmonella strains used in this study are listed in Table 1. Bacteria were grown routinely at 37 °C in Luria Bertani broth (LB) with shaking. When required, LB was supplemented with ampicillin (Amp, 100 mg l^{-1}) or kanamycin (Kan, 50 mg l^{-1}). Solid media included 15 g l^{-1} agar. When necessary, growth media was treated with menadione (50 μM).

Bioinformatic analysis

Bioinformatic analyses in search for *marsox* boxes at the *ompW* promoter region was performed using the Vector NTI software using the sequences described by Martin *et al*., (1999), and Gil *et al*., (2009).

Construction of strains and cloning

For the construction of the double mutant strains we used bacteriophage P22 HT105/1 *int* -201 using a single mutant strain as the donor and the other as the receptor (Ebel-Tsipis *et al*., 1972). The presence of substitution mutations was confirmed by PCR using specific primers (Table 2).

Genetic complementation of the Δ*soxS,* Δ*marA*, Δ*marA soxS* strains was performed using plasmid pBR322-*soxS*, pBR322-*marA,* pBR322-*rob* and pBR322-*marA*-*soxS*. To generate these plasmids, *S*. Typhimurium *soxS, marA* and *rob* genes were amplified by PCR using

primers listed in Table 2. The restriction sites (*Eco*RI and *Bam*HI, respectively) at the ends of the DNA fragment were introduced in the PCR primers (bold sequences, Table 2) and digested with the corresponding enzymes. The digested PCR product was cloned into the multiple cloning sites (MCS) of pBR322. To generate plasmid pBR322-*marA-soxS*, primers pBR322_MarAR_EcoRI_Fw with pBR322_MarAR_complsoxS_Rv and pBR322_SoxS_complmarA_Fw with pBR322_SoxS_BamHI_Rv (Table 2), were used to generate overlapping PCR products spanning the divergent construct *marA-soxS* taking advantage of the complementary sequence added (italics sequence Table 2). The resulting PCR products were used as templates in a second reaction with primers pBR322_MarAR_EcoRI_Fw and SoxS_BamHI_Rv to generate the divergent construct, which was digested and cloned into the MCS of plasmid pBR322.

RNA isolation and mRNA detection

An overnight bacterial culture was diluted 100-fold with fresh LB medium and grown at 37 °C with shaking up to an OD_{600} ~ 0.4. The culture was split into two 10 ml aliquots and one of them was incubated with 50 μM menadione. Cells were grown at 37 °C and 4 ml aliquots were withdrawn 20 min after menadione exposure. Total RNA was extracted using the GenElute Total RNA purification kit (Sigma) following the manufacturer's instructions. Total RNA was treated with 2U of DNase I to remove trace amounts of DNA. cDNA synthesis was carried out at 37 °C for 1 h in 25 μl of a mixture that contained 2.5 pmol of the specific primers, 10 μl of template RNA (5 μg), 0.2 mM dNTPs, 1 μl of sterile water, 4 μl of 5X buffer [250 mM Tris-HCl pH 8.3, 375 mM KCl, 15 mM $MgCl_2$, 10 mM DTT, 40 U of RNasin and 200 U of MMLV reverse transcriptase (Invitrogen)]. Relative quantification of the transcript levels of *ompW*, *marA*, *rob* and *soxS*

by real-time RT-PCR (qRT-PCR) was performed using the Brilliant II SYBR Green QPCR Master Reagent kit and the Mx3000P detection system (Stratagene). 16S rRNA levels were used for normalization. The qRT-PCR mixture (20 µl) contained 1 µl of cDNA template and 120 nM of each primer [ompW_RT_Fw and ompW_RT_Rv, for the *ompW* gene; marA_RT_Fw and marA_RT_Rv, for the *marA* gene; soxS_RT_Fw and soxS_RT_Rv, for the *soxS* gene; rob_RT_Fw and rob_RT_Rv, for the *rob* gene; 16S_RT_Fw and 16S_RT_Rv, for the 16S rRNA gene (16S) (Table 2)]. The qRT-PCR was performed under the following conditions: 10 min at 95 °C followed by 40 cycles of 30 s at 95 °C, 45 s at 53 °C and 30 s at 72 °C, followed by a melting cycle from 53 to 95 °C to check for amplification specificity. A standard quantification curve with serial dilutions of RT-PCR products was constructed for each gene to calculate the amplification efficiency. These values were used to obtain the ratio between the gene of interest and the expression of the 16S rRNA gene as described by Pfaffl (2001). All experiments were performed in three biological and technical replicates.

Protein purification

Briefly, *E. coli* BL21 cells harboring plasmid pET-TOPO-*soxS* or *marA* were grown in 500 ml of LB medium supplemented with amplicillin (100 µg ml^{-1}) to OD_{600} ~ 0.4 and protein overexpression was carried out by adding 1 mM IPTG and further growth for 6 h. His-tagged SoxS and MarA used in EMSAs was purified as previously described (Collao *et al*., 2012).

Immunoblot analysis

3xFLAG-containing fusion protein was detected by immunoblotting using an anti-FLAG M2 monoclonal antibody (Sigma). Strains carrying the epitope-tagged construct were grown at 37 °C with shaking up to an OD_{600} ~ 0.4. The culture was split into two 10 ml aliquots and one of them was incubated with 50 μM menadione. Cultures were grown at 37 °C and after 20 min of exposure cells were centrifuged at 10,000 *x g* for 3 min. Bacterial pellets were suspended in 100 mM Tris-HCl (pH 8.0) and subjected to 3 rounds of sonication of 30 s each. After centrifuging at 13,000 *x g* for 5 min, the pelleted material was subjected to SDS-PAGE and size-separated proteins were electroblotted onto nitrocellulose membranes, incubated with anti-FLAG Ab M2 (1:1000 dilution) upon which the FLAG epitope was detected with peroxidase-conjugated anti-mouse IgG and peroxidase activity.

Construction of transcriptional fusions with the 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_pLacZ_-600_Fw and ompW_pLacZ_+1_Rv using gDNA from *S*. Typhimurium as a template (strain 14028s). The restriction sites (*Kpn*I and *Hin*dIII, respectively) at the ends of the DNA fragment were introduced by the PCR primers (underlined sequences, Table 2) 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 p*ompW-lacZ.* To generate plasmids pMutA-*lacZ*, pMutB-*lacZ* and pMutC-*lacZ*, primers ompW_pLacZ_-600Fw with pOmpW_MUTA_Rv, pOmpW_MUTB_Rv or pOmpW_MUTC_Rv and ompW_pLacZ_+1_Rv with pOmpW_MUTA_Fw, pOmpW_MUTB_Fw or pOmpW_MUTC_Fw (Table 2) were used to generate overlapping PCR products spanning the whole length of the *ompW* promoter.

The resulting PCR products were used as templates in a second reaction with primers ompW_pLacZ_-600_Fw and ompW_pLacZ_-1_Rv to generate the mutated *ompW* promoter, which was digested and cloned into the MCS of plasmid pLacZ-Basic. Mutation of other sites was generated by the same way, generating plasmids pMutAB-*lacZ*, pMutAC-*lacZ*, pMutBC-*lacZ* and pMutABC-*lacZ*. Constructions were confirmed by DNA sequencing. The constructs were transformed into strain 14028s. To evaluate activity, cells at OD_{600} ~ 0.4 were grown for 20 min in the presence of 50 μM menadione. Control cells received no treatment. β-galactosidase activity was determined as previously described by Gil *et al*., (2007).

Electrophoretic mobility shift assay (EMSA)

To study protein binding to the promoter region of *ompW*, a non-radioactive EMSA was performed according to the protocol described by De la Cruz *et al*., (2007). The probes were obtained by PCR using specific primers pLacZ_OmpW_-600Fw and pLacZ_OmpW_+1Rv, to amplify the promoter region of *ompW* (600 bp); and pOmpW_+1_Fw with pOmpW_+130_Rv (Table 2) for the negative control encompassing the coding region of *ompW* (130 bp). PCR products used in EMSAs with mutations in the *mar*/*sox*/*rob* boxes A, B and C were generated using primers pLacZ_OmpW_-600Fw and pLacZ_OmpW_+1Rv and plasmids pMutAB-*lacZ*, pMutAC-*lacZ*, pMutBC-*lacZ* and pMutABC-*lacZ* as templates. Both, the promoter region and the negative control (~2 ng ml^{-1}) were mixed with increasing amounts of purified MarA or SoxS in the presence of binding buffer (20 mM HEPES, 100 mM KCl, 2 mM $MgCl_2$, 0.1 mM EDTA, 20 %, v/v glycerol). The mixture was incubated for 30 min at room temperature and loaded on a native 6 % polyacrylamide gel in 0.5X Tris-borate-EDTA buffer. The DNA bands were

visualized by ethidium bromide staining on a UV transilluminator. All primers used in this work were designed using the Vector NTI 10 Software.

Results

ompW is positively regulated after menadione treatment.

In order to evaluate the effect of menadione in the expression of *ompW*, we analyzed the transcript levels in a *S*. Typhimurium wild type strain treated with the toxic compound. As shown in Fig. 1a, *ompW* transcript levels were increased (20.22 ± 1.70 fold changes) as compared to control untreated cells. To correlate the changes in the levels of transcripts with the gene product OmpW (~ 21 kDa) and evaluate possible post-transcriptional regulations, we constructed translational fusions in the wild type strain and evaluated its levels as described in methods. In agreement with qRT-PCR analysis, OmpW levels were increased as compared to untreated cells (Fig. 1b).

ompW is positively regulated by MarA and SoxS.

We previously demonstrated that SoxS positively regulates *ompW* in response to paraquat (Gil *et al*., 2009). Since MarA, SoxS and Rob co-regulate several genes (Martin & Rosner, 2002), we evaluated *ompW* transcript levels in the different genetic backgrounds by qRT-PCR after exposure to menadione. As shown in Fig. 2(a), (b), in strains Δ*marA* and Δ*soxS* the transcript levels were decreased after exposure to menadione (0.27 ± 0.08 and 0.17 ± 0.01 fold changes, respectively, vs 20-fold increase in wild type cells exposed to toxic compound). Contrarily, in the *rob* mutant strain they were up-regulated to similar levels as those found in the wild type strain after the treatment (11.42 ± 0.52 fold change,

Fig. S1a). As expected, in *trans* complementation of strains Δ*marA* and Δ*soxS* restored the positive regulation observed in the wild type strain (Fig. 2a, b).

To further confirm our result, we generated translational fusions of *ompW* in the Δ*marA* and Δ*soxS* genetic backgrounds and determined the protein levels by western blot. As shown in Fig S1(a), the positive regulation observed in the wild type strain after menadione treatment was retained in the Δ*rob* strain (Fig S1b), while in strains Δ*marA* and Δ*soxS* it was abolished. Taken together, qRT-PCR and western blot analyses suggest that MarA and SoxS are required to positively regulate *ompW* in response to menadione, while Rob is not involved in this response.

MarA and SoxS bind to the *ompW* promoter region.

To evaluate if the regulation of *ompW* by MarA and SoxS was due to a direct interaction, we performed EMSAs to determine if the purified proteins were able to bind to its promoter region. The bioinformatic analysis predicted the presence of three putative *mar*/*sox*/*rob* boxes (Fig 3a), two novel ones named MS-A and MS-B, in addition to the previously identified SoxS binding site (MS-C) described by Gil *et al*., (2009). All 3 binding sites presented the two characteristic elements described at *marsox* boxes, CWA and the highly conserved GCAY (Li & Demple, 1994), which are required for the stability of the interaction and for protein binding, respectively (Li & Demple, 1996). To confirm the interactions, we performed EMSAs using a PCR product spanning the promoter region from positions -600 to +1 with respect to the transcription start site and increasing amounts of purified MarA or SoxS. As a negative control, a PCR product that included positions +1 to +130 of *ompW* was used. Both MarA and SoxS were able to bind to the wild type

promoter (Fig. 3c, d), although at different concentrations. MarA generated a change in the electrophoretic mobility at a concentration of 100 nM, while SoxS required 400 nM (Fig. 3c, d, respectively, fragment A). Mutation of the GCAY element to AAAY (Fig 3a) in the three predicted boxes required doubling the amount of both MarA and SoxS to generate a shift in the electrophoretic mobility as compared to the wild type promoter, while mutating MS-A and MS-C together completely abolished the interaction with both proteins (Fragments F and H, Fig 3c, d), suggesting that they are required for their binding *in vitro*.

The promoter region of *ompW* has two functional *marsox* boxes.

To determine which *marsox* boxes were functional *in vivo*, we constructed transcriptional fusions of the *ompW* promoter region with the fragments used for EMSAs and schematized in Fig. 3(a). The different constructions were transformed into strain 14028s and β-galactosidase activity was measured. All of the activities were compared to that of strain 14028s with the wild type construction (A). Cells containing the wild type promoter (A) or MS-B mutated (C) showed a 2 fold increase in β-galactosidase activity after exposure to the toxic compound (Fig. 3b), indicating that MS-B is dispensable for *ompW* up-regulation by MarA and SoxS in response to menadione. However, individually mutating MS-A and MS-C or together resulted in no regulation after exposure to the toxic compound (Fig. 3b, fragments B, D, E, F, G and H), indicating that both sites are required for the positive regulation by MarA and SoxS in strain 14028s, in agreement with EMSAs.

Both MarA and SoxS are required for *ompW* positive regulation.

To determine whether MarA and SoxS individually regulated *ompW* in response to menadione or if they were both required, we generated a double Δ*marA soxS* strain and

measured *ompW* transcript levels in the presence or absence of menadione. As observed in the individual mutants, *ompW* levels remained decreased in the Δ*marA soxS* strain after treatment with the toxic compound (0.29 ± 0.05 fold change, Fig. 4). When the double mutant strain was complemented in *trans* with a plasmid carrying the *S*. Typhimurium *soxS* or *marA* gene (Δ*marA soxS*/pB322-*soxS* and Δ*marA soxS*/pB322-*marA*, respectively), the transcript levels remained decreased after menadione treatment (0.354 ± 0.1 and 0.314 ± 0.06 fold change, respectively, Fig. 4). Contrarily, when the double mutant strain was complemented with a plasmid coding for both *marA* and *soxS* (Δ*marA soxS*/pB322-*marA_soxS*), the positive regulation was partially restored to similar levels as those observed in the wild type strain exposed to menadione (6.81 ± 1.13 fold change, Fig. 4), indicating that both MarA and SoxS are required for the positive regulation.

MarA and SoxS work cooperatively

Since both MarA and SoxS were required to positively regulate *ompW*, we hypothesized that both proteins might act cooperatively. To evaluate this possibility, we performed EMSAs with the *ompW* promoter mutated at MS-B (fragment C, Fig. 3a), constant amounts of either MarA (Fig. 5a) or SoxS (Fig. 5b), and increasing amounts of the corresponding counterpart. When MarA remained constant (200 nM), adding increasing amounts of SoxS, from 0.0125 to 0.8 μM, resulted in a shift of a higher molecular weight than that generated by the individual proteins (Fig. 5a). Interestingly, even at the lower concentrations of SoxS (0.125 μM) the high molecular weight complex was observed, while incubating SoxS alone required 0.4 μM to observe a shift using the same DNA probe (Fig. 3b, fragment C), suggesting that the affinity of SoxS for the promoter region of *ompW* increases in the presence of MarA. In agreement, using a constant amount of SoxS (0.8

μM) and increasing amounts of MarA (3.3 to 200 nM) resulted in a similar shift with a higher molecular weight as that observed for the individual proteins (Fig. 5b). As observed for SoxS, lower levels of MarA were required to form the high molecular weight complex (100 nM) than when the protein was incubated alone with the *ompW* promoter (200 nM), suggesting the same increased affinity for the promoter region as observed for the case of SoxS. Taken together, our results indicate that both MarA and SoxS are required to positively regulate *ompW* and that they cooperatively bind to the promoter region.

Discussion

The OmpW protein is an immunogenic 22 KDa (Jalajakumari *et al*., 1990) minor porin and has been related in osmoprotection (Hu *et al*., 2005), the efflux and resistance towards paraquat (Gil *et al*., 2009), and the influx of hydrogen peroxide and hypochlorous acid (Morales *et al*., 2012). It is regulated by diverse environmental conditions including temperature, salinity, nutrient availability, oxygen levels (Bisweswar *et al*., 2005), paraquat (Gil *et al*., 2009) and ROS (Morales *et al*., 2012), among others, and is differentially regulated at the transcriptional level by FNR (anaerobiosis), ArcA (H_2O_2 and NaOCl) and SoxS (PQ) (Bouchal *et al*., 2010; Morales *et al*., 2012; Gil *et al*., 2009).

In this work, we demonstrate that both SoxS and MarA, whose response overlap and together co-regulate over 40 genes (Aono *et al*., 1998; Martin *et al*., 1999; Martin *et al*., 2000; Martin & Rosner, 2002), were required for the positive regulation of *ompW* after menadione treatment (Fig 2 and 4). Consistent with this, both transcription factors are up-regulated in response to the toxic compound (Fig S2). In agreement, SoxS is required to regulate *ompW* in response to PQ (Gil *et al*., 2009). Our results indicate that there are 2 functional *mar/sox* boxes that are required for the positive regulation in response to MEN

(Fig. 3). These sites are located approximately 100 nt upstream to the ArcA binding site (-70 to -55) required for the negative regulation in response to H_2O_2 and NaOCl (Morales *et al*., 2012). This suggests that under the assayed conditions, ArcA is not active, since as observed in the presence of NaOCl, when ArcA, MarA and SoxS are present, *ompW* is negatively regulated. It is plausible to speculate that under those conditions ArcA could bind to the -35 element and impede binding of the sigma factor, explaining why although both MarA and SoxS are present, *ompW* is negatively regulated. In contrast, in response to menadione ArcA could be inactive allowing MarA and SoxS to exert their regulation, although this has not been evaluated.

Our results indicate that Rob is not involved in the regulation of *ompW* (Fig. S1), indicating that it is regulated in a different manner as compared to other genes that are members of the extensively studied *mar*/*sox*/*rob* regulon, as *tolC* in *E. coli*, which is positively regulated by all of them in response to salicylate, PQ and 2,2′-dipyridyl (Zhang *et al*., 2008). In addition, previous works indicate that *rob* is repressed by MarA due to steric hindrance and in *E. coli* SoxS modulates its expression in response to PQ (McMurry & Levy, 2010; Michán *et al*., 2002). Furthermore, studies in *S*. Typhimurium showed that the transcript and protein levels of *marA* and *soxS* are increased while those of *rob* lowered in a wild-type strain treated with sodium hypochlorite (Collao *et al*., 2012), suggesting a similar mechanism.

To investigate the mechanism by which MarA and SoxS regulate *ompW*, we performed EMSAs and used transcriptional fusions of the promoter region (Fig 3 and 5). Our results indicate that both proteins are required for the positive regulation and that they act in a cooperative manner (Fig. 4 and 5). In this context, several reports provide evidence that two transcription factors work cooperatively in response to the same signal, as in

Vibrio *vulnificus* were the *nan* operon is negatively regulated by CRP and NanR in presence of N-acetylmannosamine 6-phosphate (Kim *et al*., 2011). Also, in *E. coli* CRP requires the presence of RhaR to efficiently activate *rhaSR in vivo* in response to L-rhamnose (Wickstrum *et al*., 2005). Similarly, studies in *Haemophilus influenza* suggest that CRP and SiaR regulate their respective operators by simultaneously binding to an intergenic region between *nan* and *siaPT*, where SiaR functions as both a repressor and activator, using glucosamine-6-phosphate as a co-activator and interacts with CRP to regulate these divergent promoters (Johnston *et al*., 2010). Furthermore, in *Myxococcus xanthus* MrPC2 and FruA bind cooperatively to three sites at the *fmgE* promoter region, and it has been proposed that one site is necessary to recruit MrpC2 and FruA to the promoter, while the other two are required to activate it (Son B *et al*., 2011). However, most of these studies mainly show that the effect over the target genes is synergic. Contrarily, our results indicate that MarA and SoxS are required to positively modulate *ompW* expression (Fig. 4 and 5). From our knowledge this is the first report demonstrating such effect. Further studies addressing whether this is a common feature of regulating gene expression by MarA and SoxS, novel targets subject to a similar regulation and the mechanism by which these proteins interact are required, and are under study in our laboratory.

Acknowledgments

This work was financed by grant FONDECYT # 1120384 to CPS. BC and EHM work was supported by Doctoral fellowships granted by CONICYT. Additionally BC and

382 EHM work was supported by grants DI-19-12/I and DI-24-12/I, respectively, from
383 Universidad Andres Bello.

384

385 **References**

386 **Aono, R., Tsukagoshi, N. & Yamamoto, M. (1998).** Involvement of outer membrane
387 protein TolC, a possible member of the *mar-sox* regulon, in maintenance and improvement
388 of organic solvent tolerance of *Escherichia coli* K-12. *J Bacteriol* **180**, 938-944.

389 **Benz, R. & Bauer, K. (1988).** Permeation of hydrophilic molecules through the outer
390 membrane of Gram-negative bacteria. *Eur J Biochem* **176**, 1-9.

391 **Bisweswar N., Nandy R. K., Sarkar A. &Ghose A. C. (2005).** Structural features,
392 properties and regulation of the outer-membrane protein W (OmpW) of *Vibrio cholerae*.
393 *Microbiology* , **151,** 2975-2986.

394 **Bouchal, P., Struhárová, I., Budinská, E., Sedo, O., Vyhlídalová, T., Zdráhal, Z., van**
395 **Spanning, R. & Kucera, I. (2010).** Unraveling an FNR based regulatory circuit in
396 *Paracoccus denitrificans* using a proteomics-based approach. *Biochim Biophys Acta* **1804**,
397 1350-1358.

398 **Chatfield, S. N., Dorman, C. J., Hayward, C. & Dougan, G. (1991).** Role of ompR-
399 dependent genes in *Salmonella* typhimurium virulence: mutants deficient in both *ompC* and
400 *ompF* are attenuated in vivo. *Infect Immun* **59**, 449-452.

401 **Chollet, R., Bollet, C., Chevalier, J., Malléa, M., Pagès, J.-M. & Davin-Regli, A.**
402 **(2002).** *mar* operon involved in multidrug resistance of *Enterobacter aerogenes*.
403 *Antimicrob Agents Chemother* **46**, 1093-1097.
404 **Chollet, R., Chevalier, J., Bollet, C., Pages, J.-M. & Davin-Regli, A. (2004).** RamA is an
405 alternate activator of the multidrug resistance cascade in *Enterobacter aerogenes*.
406 *Antimicrob Agents Chemother* **48**, 2518-2523.
407 **Collao, B., Morales, E. H., Gil, F., Polanco, R., Calderón, I. L. & Saavedra, C. P.**
408 **(2012).** Differential expression of the transcription factors MarA, Rob, and SoxS of
409 *Salmonella* Typhimurium in response to sodium hypochlorite: down-regulation of *rob* by
410 MarA and SoxS. *Arch Microbiol* **194**, 933-942
411 **De la Cruz, MA., Fernández-Mora, M., Guadarrama, C., Flores-Valdez, MA.,**
412 **Bustamante, VH., Vázquez, A. & Calva, E. (2007).** LeuO antagonizes H–NS and StpA-
413 dependent repression in *Salmonella* enterica *ompS1*. *Mol Microbiol* **66**,727-743
414 **Ebel-Tsipis, J., Fox, M. S. & Botstein D. (1972).** Generalized transduction by phage
415 P22 in *Salmonella* typhimurium. II. Mechanism of integration of transducing DNA. *J Mol*
416 *Biol* **71**, 449-469.
417 **Fujikawa, M., Kobayashi, K. & Kozawa, T. (2012)** Direct oxidation of the [2Fe-2S]
418 cluster in SoxR protein by superoxide: distinct differential sensitivity to superoxide-
419 mediated signal transduction. *J Biol Chem*. **287**, 35702-35708.
420 **Gil, F., Ipinza, F., Fuentes, J., Fumeron, R., Villarreal, J. M., Aspée, A., Mora, G. C.,**
421 **Vásquez, C. C. & Saavedra, C. (2007).** The *ompW* (porin) gene mediates methyl viologen
422 (paraquat) efflux in *Salmonella enterica* serovar typhimurium. *Res Microbiol* **158**, 529-536.

Gil, F., Hernández-Lucas, I., Polanco, R., Pacheco, N., Collao, B., Villarreal, J. M., Nardocci, G., Calva, E. & Saavedra, C. P. (2009). SoxS regulates the expression of the *Salmonella enterica* serovar Typhimurium *ompW* gene. *Microbiology* **155**, 2490-2497.

Giró, M., Carrillo, N. & Krapp, A. R. (2006). Glucose-6-phosphate dehydrogenase and ferredoxin-NADP(H) reductase contribute to damage repair during the *soxRS* response of *Escherichia coli*. *Microbiology* **152**, 1119-1128.

Greenberg, J. T., Monach, P. A., Chou, J.H., Josephy, P. D. & Demple, B. (1990). Positive control of a global antioxidant defense regulon activated by superoxide-generating agents in *Escherichia coli*. *Proc Natl Acad Sci USA* **87**, 6181-6185.

Groisman, E. A. & Ochman, H. (1994). How to become a pathogen. *Trends Microbiol* **2**, 289-294.

Gu, M. & Imlay, J. A. (2011). The SoxRS response of *Escherichia coli* is directly activated by redox-cycling drugs rather than by superoxide. *Mol Microbiol* **79**, 1136-1150.

Hassan, H. M. & Fridovich, I. (1979). Paraquat and *Escherichia coli*. *J Biol Chem* **254**, 10846-10852.

Hong, H., Patel, D. R., Tamm, L. K. & van den Berg, B. (2006). The outer membrane protein OmpW forms an eight-stranded beta-barrel with a hydrophobic channel. *J Biol Chem* **281**, 7568-7577.

Hu, W. S., Li, P. C. & Cheng, C. Y. (2005). Correlation between ceftriaxone resistance of *Salmonella enterica* serovar Typhimurium and expression of outer membrane proteins OmpW and Ail/OmpX-like protein, which are regulated by BaeR of a two-component system. *Antimicrob Agents Chemother* **49**, 3955-3958.

Imlay, J. A. (2008). Cellular defenses against superoxide and hydrogen peroxide. *Annu Rev Biochem* **77**, 755-776.

Jair, K. W., Martin, R. G., Rosner, J. L., Fujita, N., Ishihama, A. & Wolf, R. E. (1995). Purification and regulatory properties of MarA protein, a transcriptional activator of *Escherichia coli* multiple antibiotic and superoxide resistance promoters. *J Bacteriol* **177**, 7100-7104.

Jair, K. W., Yu, X., Skarstad, K., Thöny, B., Fujita, N., Ishihama, A. & Wolf, R. E. (1996). Transcriptional activation of promoters of the superoxide and multiple antibiotic resistance regulons by Rob, a binding protein of the *Escherichia coli* origin of chromosomal replication. *J Bacteriol* **178**, 2507-2513.

Jalajakumari, M. B. & Manning, P. A. (1990). Nucleotide sequence of the gene, *ompW*, encoding a 22kDa immunogenic outer membrane protein of *Vibrio cholerae*. *Nucleic Acids Res* **18,** 2180.

Jeanteur, D., Lakey, J. H. & Pattus, F. (1991). The bacterial porin superfamily: sequence alignment and structure prediction. *Mol Microbiol* **5**, 2153-2164.

Johnston, J. W., Shamsulddin, H., Miller, A-F. & Apicella, M.A. (2010). Sialic acid transport and catabolism are cooperatively regulated by SiaR and CRP in nontypeable *Haemophilus influenza*. *BMC Microbiology* **15**, 10:240.

Kato, T., Watanabe, M. & Ohta, T. (1994). Induction of the SOS response and mutations by reactive oxygen-generating compounds in various *Escherichia coli* mutants defective in the *mutM*, *mutY* or *soxRS* loci. *Mutagenesis* **9**, 245-251.

Koebnik, R., Locher, K. P. & Van Gelder, P. (2000). Structure and function of bacterial outer membrane proteins: barrels in a nutshell. *Mol Microbiol* **37**, 239-253.

Kim, B. S., Hwang, J., Kim, M. H. & Choi, S. H. (2011). Cooperative regulation of the Vibrio vulnificus *nan* gene cluster by NanR protein, cAMP receptor protein, and N-acetylmannosamine 6-phosphate. *J Biol Chem* **286**, 40889-40899.

Krapp, A. R., Humbert, M. V. & Carrillo, N. (2011). The *soxRS* response of *Escherichia coli* can be induced in the absence of oxidative stress and oxygen by modulation of NADPH content. *Microbiology* **157,** 957-965.

Kwon, H. J., Bennik, M. H., Demple, B. & Ellenberger, T. (2000). Crystal structure of the *Escherichia coli* Rob transcription factor in complex with DNA. *Nat Struct Biol* **7**, 424-430.

Lee, P. E., Demple, B. & Barton, J. K. (2009). DNA-mediated redox signaling for transcriptional activation of SoxR. *Proc Natl Acad Sci USA* **106**, 13164-13168.

Li, Z. & Demple, B. (1994). SoxS, an activator of superoxide stress genes in *Escherichia coli.* Purification and interaction with DNA. *J Biol Chem* **269**, 18371-18377.

Li, Z. & Demple, B. (1996). Sequence specificity for DNA binding by *Escherichia coli* SoxS and Rob proteins. *Mol Microbiol* **20**, 937-945.

Martin, R. G., Gillette, W. K., Rhee, S. & Rosner, J. L. (1999). Structural requirements for *marbox* function in transcriptional activation of *mar/sox/rob* regulon promoters in *Escherichia coli*: sequence, orientation and spatial relationship to the core promoter. *Mol Microbiol* **34**, 431-441.

Martin, R. G., Gillette, W. K. & Rosner, J. L. (2000). Promoter discrimination by the related transcriptional activators MarA and SoxS: differential regulation by differential binding. *Mol Microbiol* **35**, 623-634.

Martin, R. G. & Rosner, J. L. (2002). Genomics of the *marA/soxS/rob* regulon of *Escherichia coli*: identification of directly activated promoters by application of molecular genetics and informatics to microarray data. *Mol Microbiol* **44**, 1611-1624.

493 **Martin, R. G., Bartlett, E. S., Rosner, J. L. & Wall, M. E. (2008).** Activation of the
494 *Escherichia coli marA/soxS/rob* regulon in response to transcriptional activator
495 concentration. *J Mol Biol* **380**, 278-284.
496 **Michán, C., Manchado, M. & Pueyo, C. (2002).** SoxRS down-regulation of *rob*
497 transcription. *J Bacteriol* **184**, 4733-4738.
498 **Morales, E. H., Calderón, I. L., Collao, B., Gil, F., Porwollik, S., McClelland, M. &**
499 **Saavedra, C. P. (2012).** Hypochlorous acid and hydrogen peroxide-induced negative
500 regulation of *Salmonella enterica* serovar Typhimurium *ompW* by the response regulator
501 ArcA. *BMC Microbiol* **22**,12:63.
502 **Morimyo, M. (1988).** Isolation and characterization of methyl viologen-sensitive mutants
503 of *Escherichia coli* K-12. *J Bacteriol* **170**, 2136-2142.
504 **McMurry, L. M. & Levy S. B. (2010).** Evidence that regulatory protein MarA of
505 *Escherichia coli* represses *rob* by steric hindrance. *J Bacteriol* **192**, 3977-3982.
506 **Nandi, B., Nandy, R. K., Sarkar, A. & Ghose, A. C. (2005).** Structural features,
507 properties and regulation of the outer-membrane protein W (OmpW) of *Vibrio cholerae*.
508 *Microbiology* **151**, 2975-2986.
509 **Nikaido, H. (1996).** Multidrug efflux pumps of Gram-negative bacteria. *J Bacteriol* **178**,
510 5853-5859.
511 **Pfaffl, M. W. (2001).** A new mathematical model for relative quantification in real-time
512 RTPCR. *Nucleic Acids Res* **1**, 29:e45.
513 **Pomposiello, P. J., Bennik, M. H. & Demple, B. (2001).** Genome-wide transcriptional
514 profiling of the *Escherichia coli* responses to superoxide stress and sodium salicylate. *J*
515 *Bacteriol* **183**, 3890-3902.

Rodríguez-Morales, O., Fernández-Mora, M., Hernández-Lucas, I., Vázquez, A., Puente, J. L. & Calva, E. (2006). *Salmonella enterica* serovar Typhimurium *ompS1* and *ompS2* mutants are attenuated for virulence in mice. *Infect Immun* **74**, 1398-1402.

Scandalios, J. G. (2002). Oxidative stress responses -- what have genome-scale studies taught us?. *Genome Biol* **18**, 3:REVIEWS1019.

Selke, M., Meens, J., Springer, S., Frank, R. & Gerlach, G. F. (2007). Immunization of pigs to prevent disease in humans: construction and protective efficacy of a *Salmonella enterica* serovar Typhimurium live negative-marker vaccine. *Infect Immun* **75**, 2476-2483.

Semchyshyn, H., Bagnyukova, T., Storey, K. & Lushchak, V. (2005). Hydrogen peroxide increases the activities of *soxRS* regulon enzymes and the levels of oxidized proteins and lipids in *Escherichia coli*. *Cell Biol Int* **29**, 898-902.

Son, B., Liu, Y. & Kroos, L. (2011). Combinatorial regulation by MrpC2 and FruA involves three sites in the *fmgE* promoter region during *Myxococcus xanthus* development. J Bacteriol. **193,** 2756-2766.

Storz, G. & Imlay, J. A. (1999). Oxidative stress. *Curr Opin Microbiol* **2**, 188-194.

Tobes, R. & Ramos, J. L. (2002). AraC-XylS database: a family of positive transcriptional regulators in bacteria. *Nucleic Acids Res* **30**, 318-321.

Vasil'eva S. V., Stupakova M. V., Lobysheva I. I., Mikoyan V. D., Vanin A. F. (2001). Activation of the *Escherichia coli* SoxRS-regulon by nitric oxide and its physiological donors. *Biochemistry (Mosc)* **66**, 984-948.

Weiss, M. S., Abele, U., Weckesser, J., Welte, W., Schiltz, E. & Schulz, G. E. (1991). Molecular architecture and electrostatic properties of a bacterial porin. *Science* **254**, 1627-1630.

Wickstrum, J. R., Santangelo, T. J. & Egan, S. M. (2005). Cyclic AMP Receptor Protein and RhaR Synergistically Activate Transcription from the L-Rhamnose-Responsive *rhaSR* Promoter in *Escherichia coli*. *J Bacteriol* **187**, 6708-6718.

Xu, C., Wang, S., Ren, H., Lin, X. & Wu, L. (2005). Protein analysis on the expression of outer membrane proteins of *Vibrio alginolyticus* at different sodium concentrations. *Proteomics* **5**, 3142-3152.

Zhang, A., Rosner, J. L. & Martin, R. G. (2008). Transcriptional activation by MarA, SoxS and Rob of two *tolC* promoters using one binding site: a complex promoter configuration for *tolC* in *Escherichia coli*. *Mol Microbiol* **69**, 1450-1455.

Zgurskaya, H. I. & Nikaido, H. (2000). Multidrug resistance mechanism: drug efflux across two membranes. *Mol Microbiol* **37**, 219-225.

560 **Table 1. Bacterial strains used in this study.**

Strain	Relevant characteristic(s)	Source or reference
S. Typhimurium		
14028s	Wild type	G. Mora
Δ*soxS*	*soxS*::Cam	Gil *et al*., 2009
Δ*soxS*/pBAR322-*soxS*	Δ*soxS* strain complemented with pBR322 vector carrying the *S.* Typhimurium *soxS* gene and its promoter	This work
Δ*soxS*/pBR322	Δ*soxS* strain complemented with empty pBR322vector	This work
Δ*marA*	*marA*::Kan	Collao *et al*., 2012
Δ*marA*/pBR322-*marA*	Δ*marA* strain complemented with pBR322 vector carrying the *S.* Typhimurium *marA* gene and its promoter	This work
Δ*marA*/pBR322	Δ*marA* strain complemented with empty pBR322vector	This work
Δ*rob*	*rob*::Cam	Collao *et al*., 2012
Δ*rob*/pBR322-*rob*	Δ*rob* strain complemented with pBR322vector carrying the *S.* Typhimurium *rob* gene and its promoter	This work
Δ*rob*/pBR322	Δ*rob* strain complemented with empty pBR322vector	This work
ompW-3X-FLAG	Strain carrying the epitope-tagged *ompW* gene	Gil *et al*., 2009
Δ*marA* *ompW*-3X-FLAG	*marA* mutant strain carrying the epitope-tagged *ompW* gene	This work
Δ*soxS* *ompW*-3X-FLAG	*soxS* mutant strain carrying the epitope-tagged *ompW* gene	This work
Δ*rob ompW*-3X-FLAG	*rob* mutant strain carrying the epitope-tagged *ompW* gene	This work
Δ*marA* Δ*soxS*	*marA*::Kan *soxS*::Cam	Collao *et al*., 2012
Δ*marA* Δ*soxS*/pB322-*soxS*	Δ*marA* Δ*soxS* strain complemented with pBR322 vector carrying the *S.* Typhimurium *soxS* gene	This work
Δ*marA* Δ*soxS*/pB322-*marA*	Δ*marA* Δ*soxS* strain complemented with pBR322 vector carrying the *S.* Typhimurium *marA* gene	This work
Δ*marA* Δ*soxS*/pB322-	Δ*marA* Δ*soxS* strain complemented with pBR322 vector	This work

Strain	Relevant characteristic(s)	Source or reference
marA-soxS	carrying the *S.* Typhimurium *marA* and *soxS* genes	
14028s/p*ompW-lacZ*	Wild type strain with pLacZ vector carrying *ompW* promoter	This work
14028s/pMutA-*lacZ*	Wild type strain with pLacZ vector carrying *ompW* promoter with MS-A mutant	This work
14028s/pMutB-*lacZ*	Wild type strain with pLacZ vector carrying *ompW* promoter with MS-B mutant	This work
14028s/pMutC-*lacZ*	Wild type strain with pLacZ vector carrying *ompW* promoter with MS-C mutant	This work
14028s/pMutAB-*lacZ*	Wild type strain with pLacZ vector carrying *ompW* promoter with MS-A and MS-B mutants	This work
14028s/pMutAC-*lacZ*	Wild type strain with pLacZ vector carrying *ompW* promoter with MS-A and MS-C mutants	This work
14028s/pMutBC-*lacZ*	Wild type strain with pLacZ vector carrying *ompW* promoter with MS-B and MS-C mutants	This work
14028s/pMutABC-*lacZ*	Wild type strain with pLacZ vector carrying *ompW* promoter with MS-A, MS-B and MS-C mutants	This work
E. coli		
Top10	F- *mcrA* Δ(*mrr-hsdRMS-mcrBC*) φ80*lacZ*ΔM15 Δl*acX74 nupG recA1 araD139* Δ(*ara-leu*)7697 *galE*15 *galK*16 *rpsL*(Str^R) *endA1* λ^-	Invitrogen
BL21(DE3)	F^- *ompT gal dcm lon hsdS*$_B$($r_B^- m_B^-$) λ(DE3 [*lacI lac*UV5-T7 gene 1 *ind1 sam7 nin5*])	Invitrogen
Top10/pET-*marA*	Top10 transformed with the pET-TOPO101 MarA vector carrying the *S.* Typhimurium *marA* gene	Collao *et al*., 2012
BL21(DE3)/pET-*marA*	BL21(DE3) transformed with the pET-TOPO101 MarA vector carrying the *S.* Typhimurium *marA* gene	Collao *et al*., 2012
Top10/pET-*soxS*	Top10 transformed with the pET-TOPO101 SoxS vector carrying the *S.* Typhimurium *soxS* gene	Collao *et al*., 2012
BL21(DE3)/pET-*soxS*	BL21(DE3) transformed with the pET-TOPO101 SoxS vector carrying the *S.* Typhimurium *soxS* gene	Collao *et al*., 2012

565 **Table. 2. Primers used in this study.**

Primer Name	Sequence
soxS_Ext_Fw	5’ GAACAGGTTAGCTGGTTGCT 3’
soxS_Ext_Rv	5’ GATTTTTTTTCCATAAATCG 3’
marA_Ext_Fw	5’ GTAGTTGCCATGGTTCAGCG 3’
marA_Ext_Rv	5’ TTGAGTATTTGCTCAAGAAA 3’
rob_Ext_Fw	5’ ACCTGTCACGTTGCCTAAAA 3’
rob_Ext_Rv	5’ GGGTGGTAGAAACCGCAGGG 3’
pOmpW_+1_Fw	5’ AGCAATACCAATATTTTCGCC 3’
pOmpW_+130_Rv	5’ CCGGACTGCACGCATAAAG 3’
pLacZ_OmpW_-600Fw[a]	5’ CGGGGTACCCCCGATATCGAAAATTCGCG 3’
pLacZ_OmpW_+1Rv[a]	5’ CCCAAGCTTACCCGCTCCATCGTTATGGT 3’
pOmpW_MUTA_Fw	5’ GCCTTTATCGCCAGG**AAA**ACAGGAGCAGACAAATATTTGC 3’
pOmpW_MUTA_Rv	5’ GCAAATATTTGTCTGCTCCTGT**TTT**CCTGGCGATAAAGGC 3’
pOmpW_MUTB_Fw	5’ TCGCCAGGGCAACAGGA**AAA**GACAAATATTTGCATAGCGT 3’
pOmpW_MUTB_Rv	5’ ACGCTATGCAAATATTTGTCT**TTT**CCTGTTGCCCTGGCGA 3’
pOmpW_MUTC_Fw	5’ GGAGCAGACAAATATTT**AAA**TAGCGTGAATATGTCAAAAT 3’
pOmpW_MUTC_Rv	5’ ATTTTGACATATTCACGCTA**TTT**AAATATTTGTCTGCTCC
pBR322_MarAR_EcoRI_Fw[b]	5’ **CCGGAATTC**CTAGTAGTTGCCATGGTTCA 3’
pBR322_MarAR_complsoxS_Rv[c]	5’ CCGCCGCGAGTTCGATCGCA*CTCCCAGCGATTACCGTCAAGAAACAGCGCCACGGTGGTT* 3’
pBR322_SoxS_complmar	5’

A_Fw[c]	CTCCCGTTAGCCAATCCGCT*AACCACCGTGGCGCTGTTTCTTGAC GGTAATCGCTGGGAG* 3’
pBR322_SoxS_BamHI_Rv[b]	5’ **CGCGGATCC**TTAATCATCTTCAAGCAGCC 3’
pBR322_MarA_BamHI_Rv[b]	5’ **CGCGGATCC**GAAACAGCGCCACGGTGGTT 3’
pBR322_SoxS_EcoRI_Fw[b]	5’ **CCGGAATTC**TTGACGGTAATCGCTGGGAG 3’
pBR322_Rob_BamHI_Fw[b]	5’ **CGCGGATCC**GCCCGTTTTCGCCCGGCTAA 3’
pBR322_Rob_EcoRI_Rv[b]	5’ **CCGGAATTC**AAAATATCCCCATCCTTTCA 3’
ompW_RT_Fw	5’ ATGAAAAAATTTACAGTGG 3’
OmpW_RT_Rv	5’ GAAACGATAGCCTGCCGA 3’
marA_RT_Fw	5’ TTCATAGCATTTTGGACTGG 3’
marA_RT_Rv	5’ TAGAGAATGGGCTCGTTGCT 3’
soxS_RT_Fw	5’ GCGGATGTTTCGTACGGTAA 3’
soxS_RT_Rv	5’GGTGACGGTAATCGCTGGGA 3’
rob_RT_Fw	5’ CCGCTGTCACTTGACAATGT 3’
rob_RT_Rv	5’ GTTTGCTGAGAATCGAAGCG 3’
16S_RT_Fw	5’ GTAGAATTCCAGGTGTAGCG 3’
16S_RT_Rv	5’ TTATCACTGGCAGTCTCCTT 3’

566 [a] underlined sequences indicate restriction sites for *Kpn*I or *Hin*dIII which were introduced

567 in the primers

568 [b] bold sequences indicate restriction sites for *Eco*RI or *Bam*HI introduced in the primers.

569 [c] sequences in italics represent complementary sequences added to generate overlapping

570 PCR products to produce the divergent *marA-soxS* construct as described in methods.

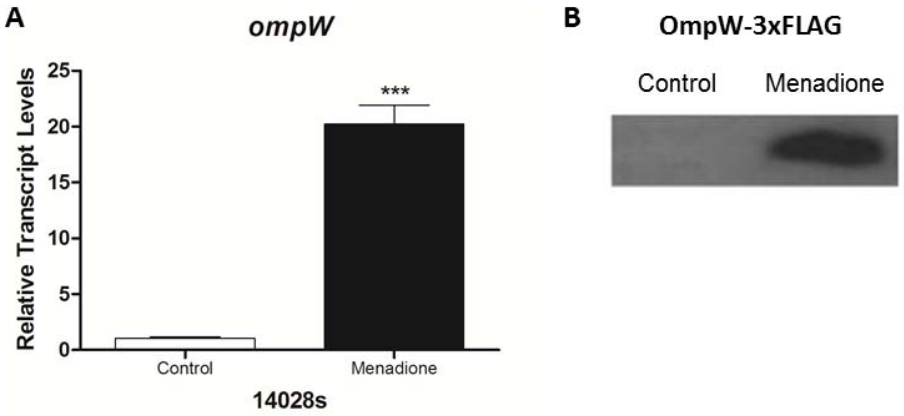
571

572

573

574

575



576

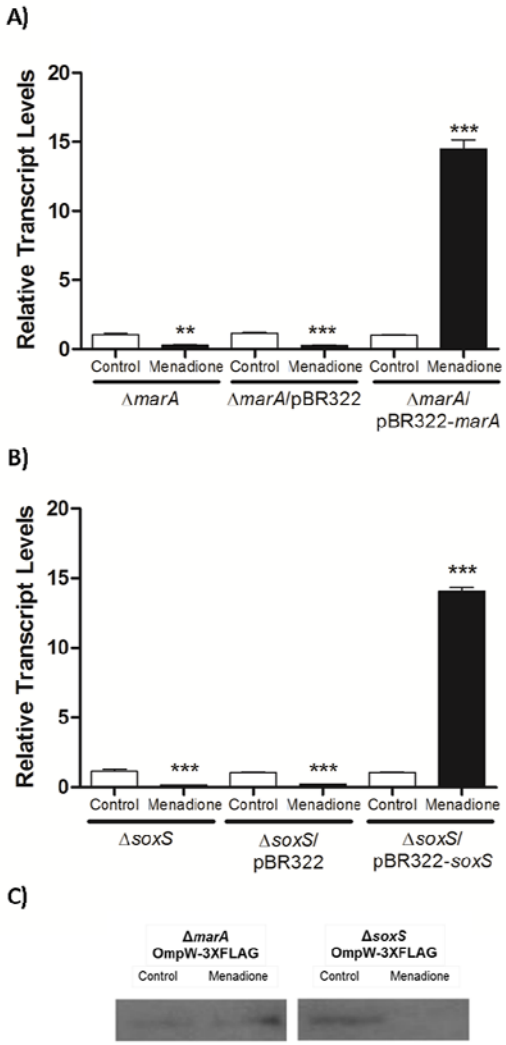
577 **Figure 1.- Effect of menadione on OmpW in *S.* Typhimurium 14028s.** Exponentially
578 growing cells were exposed to menadione (50 μM) for 20 min. Controls received no
579 treatment. **(a)** qRT-PCR was used to analyze *ompW* transcript levels from strain 14028s.
580 Values are mean ± SD. Experiments were repeated three times and asterisks represent
581 statistical differences between control and treated cells. (*** = $p < 0.001$). **(b)** OmpW-
582 3xFLAG protein was detected by western blot. Each lane was loaded with 10 μg of total
583 protein. Experiments were repeated three times and a representative result is shown.
584

585

586

587

588



589

590

591 **Figure 2.- Effect of menadione on OmpW expression in *S.* Typhimurium Δ*marA* and**
592 **Δ*soxS* strains.** Exponentially growing cells were exposed to menadione (50 μM) for 20
593 min. Controls received no treatment. **(a)** *ompW* transcripts were detected by qRT-PCR in a
594 *marA* mutant (Δ*marA*), genetically complemented strain (Δ*marA*/pBR322-*marA*) and
595 empty vector (Δ*marA*/pBR322) or **(b)** *soxS* mutant (Δ*soxS*), genetically complemented
596 strain (Δ*soxS*/pBR322-*soxS*) and empty vector (Δ*soxS*/pBR322). Experiments were
597 repeated three times and asterisks represent statistical significant differences between
598 control and treated cells for each strain. Values are mean ± SD. (** $p \leq 0.05$; *** $p \leq$
599 0.001). **(c)** OmpW-3xFLAG protein was detected in a Δ*marA*::FRT *ompW*-3XFLAG and
600 Δ*soxS*::FRT *ompW*-3XFLAG strain. Each lane was loaded with 10 μg of total protein.
601 Experiments were repeated three times and a representative result is shown.
602
603

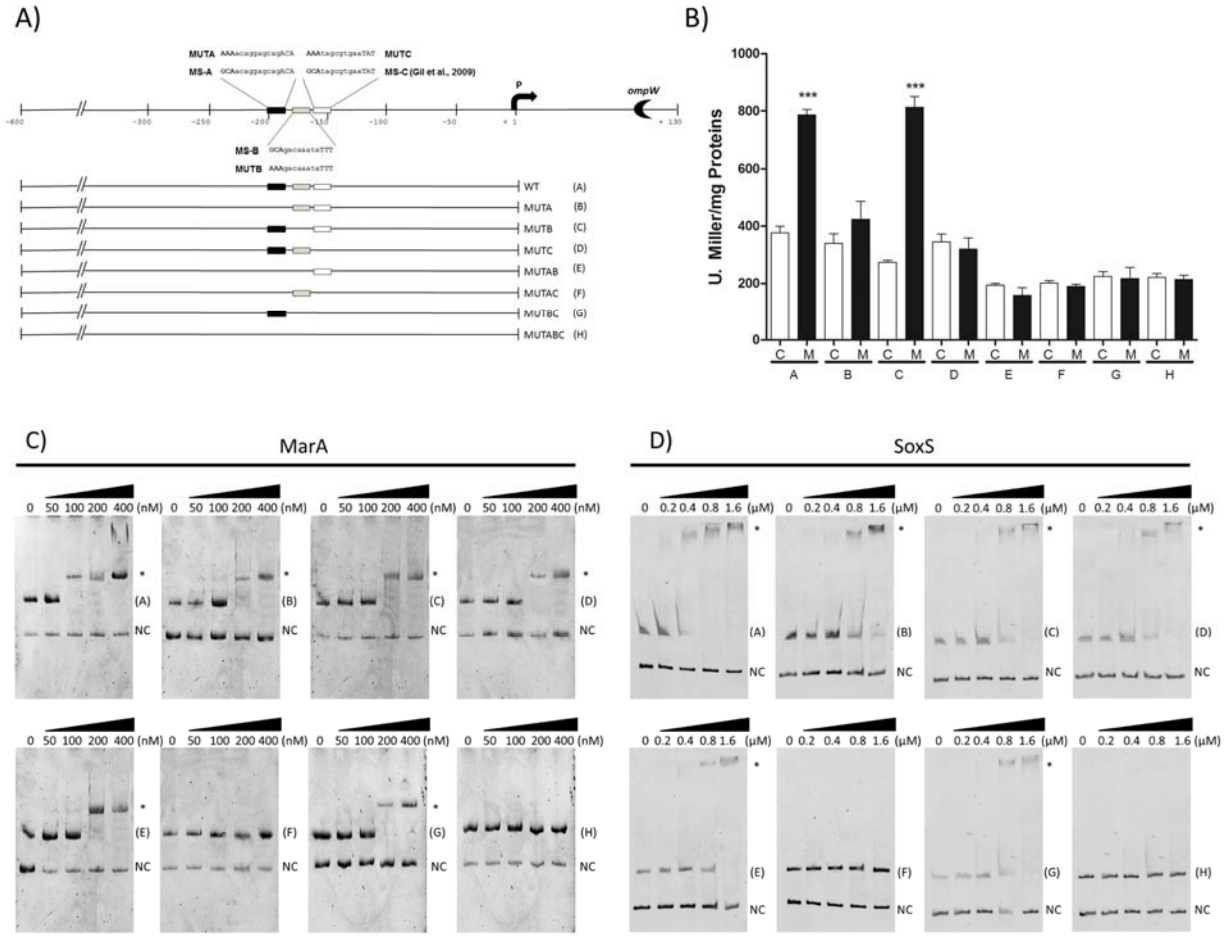


Figure 3.- Evaluating binding of MarA and SoxS at the *ompW* promoter and functionality of the *marsox* boxes.

(a) Schematic representation of *marsox* boxes MS-A (black), MS-B (gray) and MS-C (white) and substitutions generated at the *ompW* promoter (native and substituted bases are in uppercase). Marsox boxes are represented by rectangles. The name of each box is shown and represented in colors. Absence of a rectangle in the scheme represents mutation of the corresponding binding site. Name of each fragment is represented by a letter. **(b)** Expression of the wild type and mutagenized regulatory region of *ompW* in *S.* Typhimurium. Name of the construct is shown under the figure and represent the fragments shown in A. Cells were grown to OD_{600} ~ 0.4 and treated with 50 μM menadione (M) for 20 min and β-galactosidase activity was measured. Control received no treatment (C). Values represent the average of three independent experiments ± SD. (*** $p \leq 0.001$). **(c)** and **(d)** EMSA using increasing concentrations of MarA or SoxS, respectively and the fragments schematized in A. NC= negative control. The interactions were resolved by native polyacrylamide gel (6 %) electrophoresis. Bands were visualized by ethidium bromide staining.

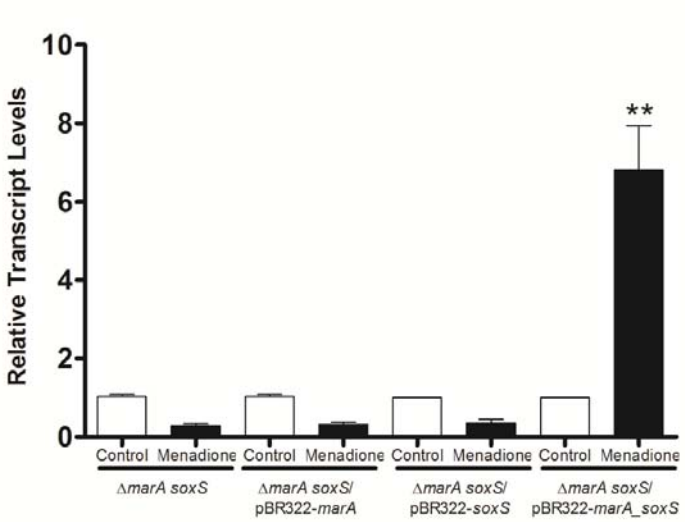


Figure 4.- Requirement of MarA and SoxS for *ompW* positive regulation. *ompW* transcript levels were measured in a double *marA soxS* mutant strain, individually complemented (Δ*marA soxS*/pBR322-*marA* and Δ*marA soxS*/pBR322-*soxS*) and complemented with both genes (Δ*marA soxS*/pBR322-*marA_soxS*). Exponentially growing cells were exposed to menadione (50 µM) for 20 min. Controls received no treatment. Experiments were repeated three times and asterisks represent statistical significant differences between control and treated cells for each strain (** $p \leq 0.005$), values are mean ± SD.

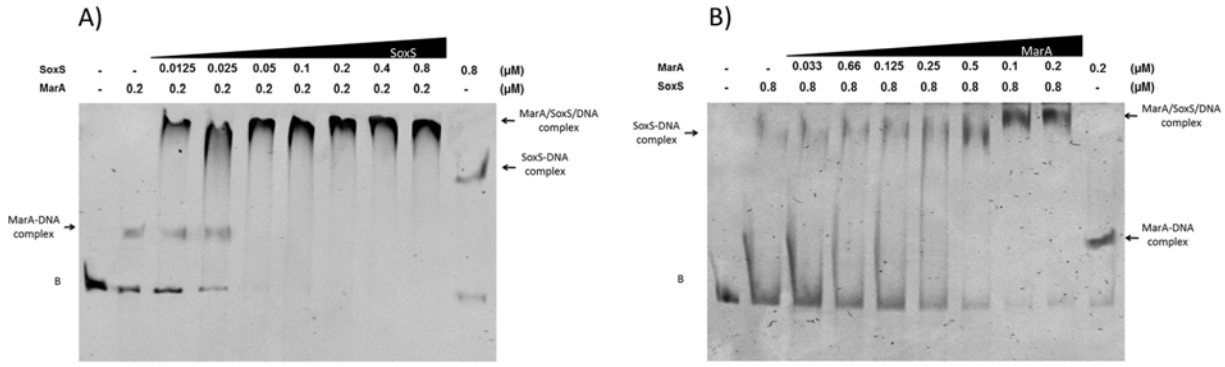


Figure 5.- Interaction of MarA and SoxS at the *ompW* promoter region. Constant amounts of purified MarA **(a)** or SoxS **(b)** were incubated with increasing amounts of SoxS or MarA, respectively, and the wild type promoter of *ompW* (fragment B). The interactions were resolved by native polyacrylamide gel (6 %) electrophoresis. Bands were visualized by ethidium bromide staining. The different DNA-protein complexes are indicated. – indicates that no protein was added.