**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; Rodriguez-Morales *et al.*, 2006). Some years ago, Morimyo (1988) isolated and characterized *Escherichia coli* mutants sensitive to paraquat, a superoxide-generating compound (Hassan & Fridovich, 1979). The deleted region in *E. coli* is highly conserved in *Salmonella enterica* serovar Typhimurium and contains the *ompW* gene (Gil *et al.*, 2007), which encodes a minor porin that has been well studied and its structure in *E. coli* and *Vibrio cholerae* has been described, 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 *Vibrio alginolyticus* high salt concentrations (NaCl 4%) induce its expression (Xu *et al.*, 2005). Furthermore, a *S. enterica* serovar 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. enterica* serovar Typhimurium 14028s *OmpW* expression is increased in the presence of paraquat and it mediates resistance (Gil *et al.*, 2007).

The cellular response to superoxide (*O₂⁻*) is regulated at the transcriptional level by the SoxRS system (Greenberg *et al.*, 1990). Upon exposure to *O₂⁻* and/or ammonium quaternary compounds, SoxR is oxidized and converted to an active form that induces the transcription of *soxS*, which binds to the promoter regions of several genes whose products are involved in the response to oxidative damage (Storz & Imlay, 1999; Scandalios, 2002; Imlay, 2008; Gu & Imlay, 2011). In this context, the evidence supports a model in which ammonium quaternary compounds are responsible for SoxR activation (Krapp *et al.*, 2011; Gu & Imlay, 2011); however, it has been recently confirmed that

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**ompW** is cooperatively upregulated by MarA and SoxS in response to menadione

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OmpW is a minor porin whose biological function has not been clearly defined. Evidence obtained in our laboratory indicates that in *Salmonella enterica* serovar Typhimurium the expression of OmpW is activated by SoxS upon exposure to paraquat and it is required for resistance. SoxS belongs to the AraC family of transcriptional regulators, like MarA and Rob. Due to their high structural similarity, the genes under their control have been grouped in the mar/sox/rob regulon, which presents 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. enterica* serovar 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 upregulated in response to menadione, while in the Δ*marA* and Δ*soxS* strains the induction was abolished. In a double Δ*marA* Δ*soxS* mutant, *ompW* transcript levels were lowered after exposure to menadione, and only complementation *in trans* with both genes restored the positive regulation. Using transcriptional fusions and electrophoretic mobility shift assays with mutant versions of the promoter region we demonstrated that two of the predicted sites were functional. Additionally, we demonstrated that MarA increases the affinity of SoxS for the *ompW* promoter region. In conclusion, our study shows that *ompW* is upregulated in response to menadione in a cooperative manner by MarA and SoxS through a direct interaction with the promoter region.

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**Abbreviations:** EMSA, electrophoretic mobility shift assay; MCS, multiple cloning site.

Two supplementary figures are available with the online version of this paper.
O$_2^-$ is also able to oxidize its 2Fe–2S cluster (Fujikawa et al., 2012). SoxS belongs to the AraC family of transcriptional regulators, of which MarA and Rob are also members (Martin & Rosner, 2002). In _E. coli_ MarA, SoxS and the N-terminal domain of Rob, which include the DNA-binding domain, share approximately 50% amino acid sequence identity (Jair et al., 1995, 1996; Tobes & Ramos, 2002). The _mar/sox/rob_ regulons overlap and together they co-regulate, by direct binding to the promoter regions, more than 40 different genes (Aono et al., 1998; Martin et al., 1999, 2000; Martin & Rosner, 2002). Expression of MarA, SoxS and Rob is increased upon exposure to a wide variety of signals. MarA is increased in response to weak acid conditions and salicylate treatment (Pomposiello et al., 2001); SoxS in response to nitric oxide, superoxide and ammonium quaternary compounds (Li & Demple, 1994; Vasil’eva et al., 2001); and Rob after treatment with bile salts and dipiridyl (Storz & Imlay, 1999; Semchyshyn et al., 2005; Kwon et al., 2000). In _E. coli_, their upregulation is correlated with changes in the expression of genes involved in the efflux of antibiotics (acrAB and tolC), decrease in outer-membrane permeability (micF), superoxide resistance (fpr and sodA), DNA repair systems (nfo) and those with unknown function (Aono et al., 1998; Pomposiello et al., 2001; Chollet et al., 2002, 2004; Girò et al., 2006; Lee et al., 2009). Due to the high structural similarity among these proteins, genes under their control have been denominated the _marsox/rob_ regulon, which present a DNA-binding consensus sequence at their promoter regions, denominated the _marsox_ box which is degenerate and asymmetrical (AYnGCAmWnnRYYAAAY), and has been detected at various locations on the chromosome of _E. coli_ (Martin et al., 2008; Aono et al., 1998; Martin et al., 1999, 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 paraquat, and a _marsox_ box has been defined at its promoter region with the sequence 5′-TTTGACATAGGTGAATATGCAAAAATTGAT-3′ (Gil et al., 2009). Since the binding sites of the members of the _mar/sox/rob_ regulon are similar, in _S. enterica_ serovar 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 on _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 _Δrob_ strains, _ompW_ was upregulated 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 regions 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 complementation in _trans_ with both genes was able to restore the positive regulation observed in the wild-type strain. In conclusion, we demonstrated 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 (LB) broth with shaking. When required, LB was supplemented with ampicillin (100 mg l$^{-1}$) or kanamycin (50 mg l$^{-1}$). Solid medium included 15 g agar l$^{-1}$. When necessary, growth medium was treated with menadione (50 μM).

**Bioinformatic analysis.** Bioinformatic analyses in search for _marsox_ boxes at the _ompW_ promoter region were performed using the Vector NTI software using the sequences described by Martin et al. (1999) and Gil et al. (2009).

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

Genetic complementation of the _ΔsoxS, ΔmarA, Δrob_ and _ΔmarA/soxS_ strains was performed using plasmids pBR322-soxS, pBR322-marA, pBR322-rob and pBR322-marA-soxS, respectively. To generate these plasmids, _S. enterica_ serovar Typhimurium _soxS, marA_ and _rob_ genes were amplified by PCR using primers listed in Table 2. The PCR was performed under the following conditions: 10 min at 95 °C, followed by 30 cycles of 30 s at 95 °C, 45 s at 55 °C and 1 min at 72 °C, and a final extension of 10 min at 72 °C. The restriction sites (_EcoRI_ and _BamHI_) at the ends of the DNA fragment were introduced in the PCR primers (sequences in bold type in Table 2) and were digested with the corresponding enzymes. The digested PCR product was cloned into the multiple cloning site (MCS) of pBR322. To generate plasmid pBR322-marA-soxS primers pBR322_MarAR_EcoRI_Fw with pBR322_MarAR_complsoxS_Rv and pBR322_SoxS_complmarA_Rv 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 (sequences in italic type in Table 2). The resulting PCR products were used as templates in a second reaction with primers pBR322_MarAR_EcoRI_Fw and pBR322_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 was grown at 37 °C with shaking to OD$_{550}$ = 0.4. The culture was split into two 10 ml aliquots and one of them was incubated with 50 μl 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
Table 1. Bacterial strains used in this study

| Strain | Relevant characteristic(s) | Source or reference |
|--------|---------------------------|---------------------|
| **S. enterica serovar Typhimurium** | | |
| 14028s | Wild-type | G. Mora, Universidad Andres Bello, Chile |
| ΔsoxS | soxS::Cam | Gil et al. (2009) |
| ΔsoxS/pBAR322-soxS | ΔsoxS strain complemented with pBR322 vector carrying the S. enterica serovar Typhimurium soxS gene and its promoter | This work |
| ΔsoxS/pBR322 | ΔsoxS strain with empty pBR322 vector | This work |
| ΔmarA | marA::Kan | Collao et al. (2012) |
| ΔmarA/pBR322-marA | ΔmarA strain complemented with pBR322 vector carrying the S. enterica serovar Typhimurium marA gene and its promoter | This work |
| Δrob | rob::Cam | Collao et al. (2012) |
| Δrob/pBR322-rob | Δrob strain complemented with pBR322 vector carrying the S. enterica serovar Typhimurium rob gene and its promoter | This work |
| Δrob/pBR322-ompW3-FLAG | Δrob strain with empty pBR322 vector | This work |
| ΔmarA ΔsoxS | ΔmarA ΔsoxS strain complemented with pBR322 vector carrying the S. enterica serovar Typhimurium soxS gene | This work |
| ΔmarA ΔsoxS/pBR322-soxS | ΔmarA ΔsoxS strain complemented with pBR322 vector carrying the S. enterica serovar Typhimurium marA gene | This work |
| ΔmarA ΔsoxS/pBR322-marA-soxS | ΔmarA ΔsoxS strain complemented with pBR322 vector carrying the S. enterica serovar Typhimurium marA and soxS genes | This work |
| 14028s/lacZ | Wild-type strain with pLacZ vector carrying ompW promoter | This work |
| 14028s/lacZ | Wild-type strain with pLacZ vector carrying ompW promoter with MS-A mutant | This work |
| 14028s/lacZ | Wild-type strain with pLacZ vector carrying ompW promoter with MS-B mutant | This work |
| 14028s/lacZ | Wild-type strain with pLacZ vector carrying ompW promoter with MS-C mutant | This work |
| 14028s/lacZ | Wild-type strain with pLacZ vector carrying ompW promoter with MS-A and MS-B mutants | This work |
| 14028s/lacZ | Wild-type strain with pLacZ vector carrying ompW promoter with MS-A and MS-C mutants | This work |
| 14028s/lacZ | Wild-type strain with pLacZ vector carrying ompW promoter with MS-B and MS-C mutants | This work |
| 14028s/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(mcr-hsdRMS-mcrBC) Δ80lacZΔM15 ΔlacX74 ΔlppG recA1 araD139 Ara-λleu7697 galE16 galK16 rpsL StrR indiv1 sim7 ninE | Invitrogen |
| BL21(DE3) | F− ompT gal dcm lon hsdS34 ΔDE3 [lacI lacUV5-T7, gene 1 inducible, sim3] | Invitrogen |
| Top10/pET-marA | Top10 transformed with the pET-TOPO101 MarA vector carrying the S. enterica serovar Typhimurium marA gene | Collao et al. (2012) |
| BL21(DE3)/pET-marA | BL21(DE3) transformed with the pET-TOPO101 MarA vector carrying the S. enterica serovar Typhimurium marA gene | Collao et al. (2012) |
| Top10/pET-soxS | Top10 transformed with the pET-TOPO101 SoxS vector carrying the S. enterica serovar Typhimurium soxS gene | Collao et al. (2012) |
| BL21(DE3)/pET-soxS | BL21(DE3) transformed with the pET-TOPO101 SoxS vector carrying the S. enterica serovar Typhimurium soxS gene | Collao et al. (2012) |
Table 2. Primers used in this study

Underlined sequences indicate restriction sites for KpnI or HindIII which were introduced in the primers. Sequences in bold type indicate restriction sites for EcoRI or BamHI introduced in the primers. Sequences in italics represent complementary sequences added to generate overlapping PCR products to produce the divergent marA-soxS construct as described in Methods.

| Primer name          | Sequence                                                                 |
|----------------------|---------------------------------------------------------------------------|
| soxS_Ext_Fw          | 5’-GAACAGGTATAGCTGGTTTGC-3’                                                |
| soxS_Ext_Rv          | 5’-GATTAAAAAATCCCCCATC-3’                                                 |
| marA_Ext_Fw          | 5’-GTAGCTGGCATAGCTGGTGCG-3’                                                |
| marA_Ext_Rv          | 5’-TTGAGTTTCTGTAATGAA-3’                                                  |
| rob_Ext_Fw           | 5’-ACCTGTCACTGGTTCCAA-3’                                                  |
| rob_Ext_Rv           | 5’-GGGTTGTAGAAACCGCCAG-3’                                                 |
| pOmpW_+1_Fw          | 5’-AGCAATACCAATTATTTCCG-3’                                                |
| pOmpW_+130_Rv        | 5’-CCGGAATGCAGCCATCATAGA-3’                                               |
| pOmpW_–600Fw         | 5’-GGGATTCCCCGATATGTCGGA-3’                                               |
| pOmpW_+1Fw           | 5’-CCCAGCTTTAACCCTGTGGTTTGGT-3’                                           |
| pOmpW_MUTA_Fw        | 5’-GGACTTATGGCCAGAAGACGCGAGCGACGTA-3’                                    |
| pOmpW_MUTA_Rv        | 5’-GCAAATTGTGCTCCTGGTGCTGTTTGC-3’                                        |
| pOmpW_MUTB_Fw        | 5’-TGCCAGGACCAACGAAAGACAAATTTCTGATACGTCGAT-3’                            |
| pOmpW_MUTB_Rv        | 5’-ACCGTATGGCCATTATGGTCGTCGTG-3’                                         |
| pOmpW_MUTC_Fw        | 5’-GAGGACGACACAATTATTTCTG-3’                                              |
| pOmpW_MUTC_Rv        | 5’-ATTGGACATTATCGGCTATTTG-3’                                              |
| pBR322_MarAR_EcoRI_Fw| 5’-CCCGATTCCATGTTTCGCAATGCTGGA-3’                                       |
| pBR322_MarAR_complsoxS_Rv | 5’-CCCGGCGAGTGTCGAGGCGACGCG-3’                                      |
| pBR322_SoxS_complmarA_Fw | 5’-CTCGGATTCCATGTTTCGCAATGCTGGA-3’                                      |
| pBR322_SoxS_BamHI_Rv | 5’-CCGGGATCTTAAATCATTTCAACGACG-3’                                       |
| pBR322_Mara_BamHI_Rv | 5’-CCGGGATCTTAAATCATTTCAACGACG-3’                                       |
| pBR322_SoxS_EcoRI_Fw | 5’-GGTGAATCTGGGATATTGACG-3’                                              |
| pBR322_RobBamHI_Fw   | 5’-CCGGGATCTTAAATCATTTCAACGACG-3’                                       |
| pBR322_RobEcoRI_Fw   | 5’-GGTGAATCTGGGATATTGACG-3’                                              |
| ompW_RTC_Fw          | 5’-ATGAAAAATTTAGCATGG-3’                                                  |
| ompW_RTC_Rv          | 5’-GAAACAATTAGGCGCCTGG-3’                                                 |
| marA_RTC_Fw          | 5’-TTCATAGACTGTTTGGACATG-3’                                               |
| marA_RTC_Rv          | 5’-TAGAGATTTGGCCTGTTCGT-3’                                                |
| soxS_RTC_Fw          | 5’-GCCGATTTGTCTGATGACATG-3’                                               |
| soxS_RTC_Rv          | 5’-GGTGACGCTTAATGGTGCCG-3’                                                |
| rob_RTC_Fw           | 5’-CCGCCGTCACCTTGCACTATGGT-3’                                            |
| rob_RTC_Rv           | 5’-GTTGTTCGAGAATCAGGAAGGC-3’                                              |
| 16S_RTC_Fw           | 5’-GTGAATTCCAGGTGTACG-3’                                                  |
| 16S_RTC_Rv           | 5’-TTATCAGTGGCCAGTCTCCTT-3’                                               |

Instructions. Total RNA was treated with 2 U 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 template RNA (6 µg), 0.2 mM dNTPs, 1 µl sterile water, 4 µl 5 X buffer [250 mM Tris/Cl pH 8.3, 375 mM KCl, 15 mM MgCl₂, 10 mM DTT, 40 µl RNasin and 200 U MMLV reverse transcriptase (Invitrogen)]. Relative quantification of the transcript levels of the 16S rRNA gene 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 cDNA template and 120 nM of each primer [ompW_RTC_Fw and ompW_RTC_Rv for the ompW gene, marA_RTC_Fw and marA_RTC_Rv for the marA gene, soxS_RTC_Fw and soxS_RTC_Rv for the soxS gene, rob_RTC_Fw and rob_RTC_Rv for the rob gene, and 16S_RTC_Fw and 16S_RTC_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 15 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 harbouring plasmid pET-TOPO-soxS or marA were grown in 500 ml LB medium supplemented with ampicillin (100 µg ml⁻¹) to OD₆₀₀ ~0.4 and protein overexpression was carried out by adding 1 mM IPTG with further growth for 6 h. His-tagged SoxS and MarA used in EMSAs were purified as previously described (Collao et al., 2012).

Immunoblot analysis. Immunoblotting using an anti-FLAG M2 mAb (Sigma) detected a 3 × FLAG-containing fusion protein. Strains carrying the epitope-tagged construct were grown at 37 °C with shaking to OD₆₀₀ ~0.4. The culture was split into two 10 ml aliquots, one of which was incubated with 50 µM menadione. Cultures were
grown at 37 °C and after 20 min of exposure cells were centrifuged at 10,000 g for 3 min. Bacterial pellets were suspended in 100 mM Tris/ HCl (pH 8.0) and subjected to three rounds of sonication of 30 s each. After centrifuging at 13,000 g for 5 min, the pellet material was subjected to SDS-PAGE and size-separated proteins were electroblotted onto nitrocellulose membranes, incubated with anti-FLAG antibody 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 pLacZ_OmpW−600_Fw and pLacZ_OmpW−1_Rv using genomic DNA from S. enterica serovar Typhimurium as a template (strain 14028s). The restriction sites (KpnI and HindIII, respectively) at the ends of the DNA fragment were introduced by the PCR primers (underlined sequences in Table 2) and were digested with the corresponding enzymes. The digested PCR product was cloned into the MCS of the β-galactosidase reporter vector pLacZ Basic (GenBank accession no. U13184) (Clontech), generating plasmid pompW_lacZ. To generate plasmids pMutAB_lacZ, pMutB_lacZ and pMutC_lacZ, primers ompW_pLacZ−600_Fw 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 PCR was performed under the following conditions: 5 min at 95 °C, followed by 10 cycles of 30 s at 94 °C, 30 s at 40 °C and 2 min at 72 °C, followed by 10 cycles of 30 s at 94 °C, 30 s at 45 °C and 2 min at 72 °C, and 20 cycles of 30 s at 94 °C, 30 s at 50 °C and 2 min at 72 °C, and a final extension of 10 min at 72 °C. The resulting PCR products were used as templates in a second reaction with primers pLacZ_OmpW−600_Fw and pLacZ_OmpW−1_Rv to amplify the mutated ompW promoter, which was digested and cloned into the MCS of plasmid pLacZ Basic. Conditions were generated in Methods. In agreement with qRT-PCR analysis, OmpW levels were increased as compared with those of untreated cells (Fig. 1b).
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To further confirm our result, we generated translational fusions of ompW in the ΔmarA and ΔsoxS genetic backgrounds and determined the protein levels by performing a Western blot. As shown in Fig. S1(a), the positive regulation observed in the wild-type strain after menadione treatment was retained in the Δrob strain (Fig. S1b), while in ΔmarA and ΔsoxS strains it was abolished (Fig. 2a–c). 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 three 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, with increasing concentrations of purified MarA or SoxS. As a negative control, a PCR product that included a region from +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 AAAAY (Fig. 3a) in the three predicted boxes required doubling of the amount of both MarA and SoxS to generate a shift in the electrophoretic mobility as compared with that of 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 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, schematized in Fig. 3(a). The different constructs were transformed into strain 14028s and β-galactosidase activity was measured. All activities were compared with that of

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**Fig. 2.** Effect of menadione on OmpW expression in *S. enterica* serovar Typhimurium ΔmarA and ΔsoxS strains. Exponentially growing cells were exposed to menadione (50 μM) for 20 min. Controls received no treatment. ompW transcripts were detected by qRT-PCR in a marA mutant, ΔmarA, genetically complemented strain ΔmarA/pBR322–marA and a strain carrying the empty vector, ΔmarA/pBR322 (a), and in a soxS mutant, ΔsoxS, genetically complemented strain ΔsoxS/pBR322–soxS and a strain carrying the empty vector, ΔsoxS/pBR322 (b). Experiments were repeated three times and asterisks represent significant differences between control and treated cells for each strain. Values are means ± SD (***P < 0.005). (c) OmpW-3×FLAG protein was detected in a ΔmarA::FRT ompW-3×FLAG and ΔsoxS::FRT ompW-3×FLAG strain. Each lane was loaded with 10 μg total protein. Experiments were repeated three times and a representative result is shown.
strain 14028s with the wild-type construct (A). Cells containing the wild-type promoter (A) or MS-B mutated (C) showed a twofold increase in β-galactosidase activity after exposure to the toxic compound (Fig. 3b), indicating that MS-B is dispensable for ompW upregulation by MarA and SoxS in response to menadione. However, individually mutating MS-A and MS-C or mutating both 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, results which are in agreement with those from 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 ΔmarA 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 ΔmarA 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

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MarA and SoxS work cooperatively

Since both MarA and SoxS were required to positively regulate ompW, we hypothesized that the two 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 to a higher molecular mass than that generated by the individual proteins (Fig. 5a). Interestingly, even at the lower concentrations of SoxS (0.0125 μM) the high-molecular-mass complex was observed, while incubating with SoxS alone required 0.4 μM to produce 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 with this, using a constant amount of SoxS (0.8 μM) and increasing amounts of MarA (33–200 nM) resulted in a similar shift with a higher molecular mass to that observed for the individual proteins (Fig. 5b). As observed for SoxS, lower levels of MarA were required to form the high-molecular-mass 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 in 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 & Manning, 1990) minor porin and has been related to 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 (Nandi et al., 2005), paraquat (Gil et al., 2009) and reactive oxygen species (Morales et al., 2012), among others, and is differentially regulated at the transcriptional level by FNR (anaerobiosis), ArcA (H2O2 and NaOCl) and SoxS (paraquat) (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, 2000; Martin & Rosner, 2002), were required for the positive regulation of ompW after menadione treatment (Figs 2 and 4). Consistent with this, both transcription factors are upregulated in response to the toxic compound (Fig S2), and SoxS is required to regulate ompW in response to paraquat (Gil et al., 2009). Our results indicate that there are two functional mar/sox boxes that are required for the positive regulation in response to menadione (Fig. 3). These sites are located approximately 100 nt upstream from the ArcA-binding site (from −70 to −55), required for the negative regulation in response to H2O2 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 with other genes that are members of the extensively studied *mar*/*sox*/*rob* regulon, like *tolC* in *E. coli*, which is positively regulated by all of them in response to salicylate, paraquat and 2,2'-dipyridyl (Zhang et al., 2008). In addition, previous work indicates that *rob* is repressed by MarA due to steric hindrance and in *E. coli* SoxS modulates its expression in response to paraquat (McMurry & Levy, 2010; Michán et al., 2002). Furthermore, studies in *S. enterica* serovar Typhimurium showed that the transcript and protein levels of *marA* and *soxS* are increased, while those of *rob* are 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 (Figs 3 and 5). Our results indicate that both proteins are required for positive regulation and that they act in a cooperative manner (Figs 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*, where the *nan* operon is negatively regulated by CRP and NanR in the 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 influenzae* 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 *fingE* 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 et al., 2011). However, most of these studies mainly show that the effect on the target genes is synergic. In contrast, our results indicate that MarA and SoxS are required to positively modulate *ompW* expression (Figs 4 and 5). To our knowledge, this is the first report demonstrating such an effect. Further studies addressing whether this is a common feature of regulation of gene expression by MarA and SoxS, novel targets subject to similar regulation, and the mechanism by which these proteins interact are under examination in our laboratory.

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