SoxS regulates the expression of the *Salmonella enterica* serovar Typhimurium *ompW* gene

F. Gil,1 I. Hernández-Lucas,2 R. Polanco,3 N. Pacheco,1 B. Collao,1 J. M. Villarreal,1 G. Nardocci,1 E. Calva2 and C. P. Saavedra1

1Laboratorio de Microbiología Molecular, Departamento de Ciencias Biológicas, Universidad Andrés Bello, Santiago, Chile
2Departamento de Microbiología Molecular, Instituto de Biotecnología, Universidad Nacional Autónoma de México, Cuernavaca, Mexico
3Laboratorio de Bioquímica, Departamento de Ciencias Biológicas, Universidad Andrés Bello, Santiago, Chile

OmpW of *Salmonella enterica* serovar Typhimurium has been described as a minor porin involved in osmoregulation, and is also affected by environmental conditions. Biochemical and genetic evidence from our laboratory indicates that OmpW is involved in efflux of and resistance towards paraquat (PQ), and its expression has been shown to be activated in response to oxidative stress. In this study we have explored *ompW* expression in response to PQ. Primer extension and transcriptional fusions showed that its expression was induced in the presence of PQ. *In silico* analyses suggested a putative binding site for the SoxS transcriptional factor at the *ompW* regulatory region. Electrophoretic mobility shift assays (EMSA) and footprinting experiments showed that SoxS binds at a region that starts close to −54 and ends at about −197 upstream of the transcription start site. Transcriptional fusions support the relevance of this region in *ompW* activation. The SoxS site is in the forward orientation and its location suggests that the *ompW* gene has a class I SoxS-dependent promoter.

INTRODUCTION

Porins are outer-membrane proteins that form pores, aqueous channels that allow passive diffusion of hydrophilic solutes, nutrients or antibacterial toxins through the bacterial outer membrane. Thus, they 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 (PQ), a superoxide-generating compound (Hassan & Fridovich, 1979). The deleted region in these *E. coli* mutants is highly conserved in *Salmonella enterica* serovar Typhimurium (S. Typhimurium) containing the *ompW* gene, which encodes a minor porin that participates in PQ resistance (Gil et al., 2007). The cellular response to superoxide is regulated at the transcriptional level by the SoxRS regulon. The regulation of the SoxRS regulon is mediated by the conversion of the SoxR protein to an active form, which induces the transcription of *soxS*, with the later binding of the SoxS protein to the promoters of many genes that take part in the response to oxidative damage. Besides SoxR, other transcriptional regulators modulate the expression of antioxidant genes in bacteria, indicating a complexity and connectivity of overlapping regulatory pathways (Storz & Imlay, 1999; Scandalios, 2002; Imlay, 2008).

OmpW is a small outer-membrane protein described in various enterobacterial species, including *E. coli*, *S. Typhimurium* and *Vibrio cholerae*, among others. Recent studies have indicated that *ompW* expression is regulated by certain environmental factors. Proteomic analysis of *Stenotrophomonas* sp. OK-5 revealed that OmpW is induced significantly in the presence of trinitrotoluene (TNT) (Ho et al., 2004). In *V. cholerae* it has been reported that OmpW expression is affected by environmental factors such as temperature, salinity, and the availability of nutrients and oxygen (Nandi et al., 2005). A putative osmoregulation function has been suggested for OmpW, since growth of *Vibrio alginolyticus*, in the presence of high concentrations of salt (4 % NaCl), results in a dramatic induction of OmpW (Xu et al., 2005).

Abbreviations: CAT, chloramphenicol acetyltransferase; EMSA, electrophoretic mobility shift assay; PQ, paraquat; ROS, reactive oxygen species.
The expression of the S. Typhimurium ompW gene increases in the presence of PQ (Gil et al., 2007). This observation agrees with other reports for other OmpW-like systems under stress, suggesting that members of this family could be regulated by several external factors, such as oxidative stress. In this study the regulation of ompW expression by PQ was evaluated. The transcription start site and SoxS-binding sites were identified in the promoter region of the ompW gene. Its location is consistent with a class I promoter, in which SoxS binds upstream of the –35 site in the forward orientation (Martin et al., 2000).

METHODS

Bacterial strains and culture conditions. S. Typhimurium strain 14028s was grown in LB (tryptone 10 g, yeast extract 5 g, NaCl 10 g per litre). When required, the following antibiotics were added (µg ml⁻¹): kanamycin (Km; 30), tetracycline (Tc; 12) and ampicillin (Amp; 100). S. Typhimurium and E. coli strains were grown aerobically at 37 °C.

DNA manipulations. Primers for PCR amplification were provided by Integrated DNA Technologies (IDT) and are listed in Table 1. Restriction enzymes, ligases, kinases, nucleotides and polymerases were obtained from New England Biolabs or Invitrogen. For sequencing, dsDNA was purified with the High Pure Plasmid Purification Kit (Roche) and sequenced using an automatic Perkin Elmer/Applied Biosystems 377-18 system.

Construction of transcriptional reporter fusions. Oligonucleotides (Table 1) were designed to amplify by PCR fragments of 500, 400, 300, 250, 200 and 170 bp (see Fig. 3) that contained 100 bp upstream and up to 400 bp upstream of the theoretical ATG of the ompW gene. Each PCR product was independently double-digested with BamHI/KpnI and ligated to pKK232-8-9-170 to -500. Fusions were sequenced in order to verify the correct DNA sequence of each construct.

Chloramphenicol acetyltransferase (CAT) assay. The CAT assay was performed as follows. S. Typhimurium was grown in LB medium supplemented with Amp or Km to OD₆₀₀ 1.0. Bacterial cultures (1.5 ml) were collected by centrifugation and washed with 0.8 ml Tris-borate-EDTA buffer. The DNA bands were visualized by agarose gel (1.5 %) electrophoresis. Five to twenty micrograms of total RNA were denatured at 95 °C for 3 min and then slowly cooled to 45 °C. The RNA was annealed with the reverse [γ-³²P]ATP-labelled primer. The primer was extended with reverse transcriptase at 42 °C for 1 h, and then the extended product was purified by protein precipitation and analysed by electrophoresis in an 8% polyacrylamide/8 M urea gel alongside sequencing ladders. Sequencing ladders were generated from a plasmid that contained 500 bp of the ompW promoter region.

Purification of His–SoxS protein. Cloning of the soxS gene in the expression vector pBAD TOPO TA was carried out according to the manufacturer’s instructions (Invitrogen). The recombinant plasmid was transformed in electrocompetent E. coli Top10 cells, and the presence of the cloned insert was confirmed by PCR and sequencing. SoxS was purified from a bacterial culture containing the cloned gene that was grown overnight in LB medium. A 100 µl volume of this preculture was inoculated in 500 ml LB medium to OD₆₀₀ 0.6 and induced for 5 h with 1 mM l-arabinose. Aliquots of 45 ml were collected and centrifuged at 4500 r.p.m. (Eppendorf 5702R centrifuge) for 30 min at 4 °C. The cell pellet was washed twice with 0.85% NaCl and suspended in 10 ml binding buffer (20 mM Tris/ HCl 0.2 M NaCl, 30 mM imidazole, pH 7.2). The cell suspension was disrupted using a sonic disruptor (Microson ultrasonic cell disruptor XL, Misonix). The suspension was centrifuged at 11 000 r.p.m. for 10 min at 4 °C to pellet cell debris. The supernatant was loaded onto a 5 ml nickel affinity chromatography column (HisTrap HP, GE Healthcare).

The column was washed and equilibrated with binding buffer before elution loading, and washed with 50 ml binding buffer. The protein was eluted with 15 ml elution buffer (10 mM Tris/HCl, 200 mM NaCl, 500 mM imidazole, pH 7.2). Fractions were monitored at 280 nm and resolved by SDS-PAGE. Finally, to obtain a highly purified protein, the band of interest was electroeluted using a Whole Gel Eluter (Bio-Rad).

<p>| Table 1. Primers used in this study |</p>
<table>
<thead>
<tr>
<th>Primer</th>
<th>Nucleotide sequence (5’–3’)</th>
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<tbody>
<tr>
<td>W500F1</td>
<td>CGgagttccAAAGGAGACAGCAAAATAGG</td>
</tr>
<tr>
<td>W500R2</td>
<td>CGggtaccGGCTCAGGCTGGCATAAAG</td>
</tr>
<tr>
<td>W400F1</td>
<td>CGgagttccAACAGGAAATATTCTGTCCC</td>
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<tr>
<td>W300F1</td>
<td>CGggtaccGGCAGCAAAATTTTGCAT</td>
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<td>W250F1</td>
<td>CGgagttccCTTAATACACGAGTTCCTCAA</td>
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<td>W200F1</td>
<td>CGgagttccATGGTTAATATAGGAAAC</td>
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<tr>
<td>W170F1</td>
<td>CGgagttccTATAGATCTATTATTATT</td>
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<td>WR</td>
<td>ACCGGCTCTATCCTTGTG</td>
</tr>
<tr>
<td>soxSF</td>
<td>CAGCCGCGTACGACGTAAG</td>
</tr>
<tr>
<td>soxSR</td>
<td>ATGTCGCCATCGACGATAAT</td>
</tr>
</tbody>
</table>
DNA fragments encompassing the promoter region of the *ompW* gene were amplified by PCR using end-labelled primer WR. Binding of recombinant SoxS to the labelled promoter fragment was performed at room temperature for 30 min in a reaction buffer containing 20 mM HEPES (pH 7.9), 100 mM KCl, 2 mM MgCl2, 0.1 mM EDTA, 0.1 mM DTT and 20 %, v/v, glycerol. The reaction mixture was treated with 0.05 U DNase I for 1.5 min. A phenol/chloroform/isoamyl alcohol extraction was carried out to extract the protein, and then the DNA was precipitated with 100 % cold ethanol. Digested DNA fragments were resolved by electrophoresis in 8 % polyacrylamide/8 M urea gels alongside sequencing ladders. Sequencing ladders were generated with the same labelled primer used for PCR amplification of the regulatory region of the *ompW* gene, using the *f* mol kit (Promega).

**RESULTS**

**Identification of the *ompW* transcription start site by primer extension**

Primer extension experiments were performed to define the *ompW* promoter region. *S. Typhimurium* 14028s transformed with the pKK232-9 250 plasmid was grown in LB medium to stationary phase, and total RNA was isolated.

Results showed that *ompW* possesses a transcription start site 30 nt upstream of the theoretical ATG (Fig. 1). Since, in the presence of PQ, transcription of the *ompW* gene was increased, consistent with our previous observations (Gil et al., 2007). To support these results, a bioinformatic search was performed using the Softberry BPROM program (www.softberry.com/berry.phtml?Topic=bprom). Thus, a 400 bp region upstream of the start codon was analysed for putative –35 and –10 promoter regions. Hence, a promoter was predicted 30 nt upstream of the ATG, in agreement with the primer extension assay (Fig. 1).

Because of increased *ompW* transcription under PQ-elicited oxidative stress (Gil et al., 2007), we also looked for potential binding sites for transcriptional activators that respond to oxidative stress, specifically to superoxide-generating agents. The putative SoxS-binding site was predicted by comparison with consensus sequences described for genes that are activated by this factor (Fig. 2). This site was located between nucleotides –130 and –159 with respect to the transcription start site (Fig. 3, black rectangle). This suggests that *ompW* expression is regulated by the concentration of intracellular reactive oxygen species (ROS), particularly by superoxide.

To define the role of SoxS in the regulation of *ompW* expression and to confirm the activity of the putative promoter, we constructed transcriptional fusions encompassing different lengths of the *ompW* promoter region (Fig. 3). These were then cloned into plasmids pKK232-8OTc or pKK232-9OTc, which contain a promoterless cat reporter gene. Plasmids were transformed into S. Typhimurium 14028s to measure CAT specific activity, either in the absence or in the presence of 4 mM PQ (Fig. 4).

The activity of the reporter was negligible for the shortest CAT fusions (data not shown), demonstrating a very low transcriptional activity. However, the 250 bp fusion (pKK232-250) showed increased CAT activity in the presence of PQ (Fig. 4). Furthermore, fusions including longer upstream fragments (pKK232-300, -400 and -500) showed a similar increase in CAT activity.

To verify that *ompW* transcriptional activation was dependent on SoxS, a ΔsoxS strain was constructed by the method described by Datsenko & Wanner (2000). Plasmid pKK232-8 carrying the cat fusions with the 250 bp (ΔsoxS pKK232-250) and 500 bp (ΔsoxS pKK232-500) upstream fragments was introduced into the ΔsoxS strain. The same CAT activity was observed with and without PQ for both constructs (Fig. 4). These results support the notion that the activation of *ompW* is SoxS-dependent.

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**Fig. 1.** Determination of the transcriptional start site of *ompW*. A primer extension assay was performed using total RNA extracted from a culture of *S. Typhimurium* grown in the presence (+) or absence (–) of 4 mM PQ. The 5’-32P-labelled primer W500R2 was used to obtain the respective cDNA. cDNA was resolved in a sequencing gel together with a sequencing reaction performed with the same primer using as template a 500 bp fragment of the *ompW* promoter cloned into the pKK232-9 vector.
SoxS binds to the regulatory region of the \textit{ompW} gene

The transcriptional fusions showed that SoxS is a genetic element involved in the regulation of \textit{ompW}. Accordingly, EMSAs were performed in order to elucidate whether SoxS interacts directly with the 5' upstream regulatory region. Fig. 5 shows EMSA experiments for the different fragments described in Fig. 3, where changes in electrophoretic mobility were only observed for fragments of 500, 400 (data not shown), 300 and 250 bp in length (Fig. 5c, d). The smaller fragments of 170 and 200 bp were not shifted by SoxS (Fig. 5a, b), in agreement with the transcriptional fusion results.

DNase I protection studies

DNase I footprinting was performed using a 400 bp region upstream of the theoretical ATG and primer WR (Table 1). Purified, recombinant His6–SoxS showed a protection pattern in a dose-dependent manner, which began close to −54 and ended at position −197 upstream of the transcriptional start site and contained, between −130 and −159, the predicted soxbox (Fig. 6). This finding is consistent with the transcriptional fusion and EMSA results, and further proves the sequence recognized by SoxS. We conclude from these results that the \textit{ompW} promoter is a class I promoter in which the soxbox lies upstream of the −35 element in the forward orientation in

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{soxsbinding.png}
\caption{Bioinformatic analysis of promoter regions containing SoxS binding sites. Alignment of a putative SoxS binding site from the \textit{ompW} promoter of \textit{S. Typhimurium} 14028s with three SoxS consensus sequences reported for other bacteria. Conserved nucleotides in the different sequences are highlighted by a grey background. The bottom of the figure shows, as a result of alignment, a consensus sequence that contains two motifs, a GCAY motif (SoxS binding) and a CWA motif (DNA–SoxS interaction stability).}
\end{figure}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{fig3.png}
\caption{Schematic representation of \textit{ompW} and fragments used in reporter constructions. Schematic representation of \textit{ompW} regulatory region showing the +1 transcribed nucleotide, as experimentally determined (see Fig. 1). Also shown are a putative SoxS binding site (black rectangle) and that obtained by DNase I footprinting assay (white rectangle; see Fig. 6). PCR fragments of different lengths used for reporter analysis (see Fig. 4) and EMSA (see Fig. 5) are shown.}
\end{figure}
DISCUSSION

The ompW gene of S. Typhimurium encodes a predicted 192 residue 24 kDa outer membrane pore-forming protein, which mediates efflux and resistance to PQ (Gil et al., 2007). Its transcriptional profile suggests that ompW expression is activated by superoxide stress. Results from this work confirm and extend the role of SoxS in the regulation of genes that collectively aid in avoiding and/or repairing the damage caused by oxidizing agents (Greenberg et al., 1990; Pomposiello et al., 2003). The SoxS regulon is also involved in the positive regulation of other outer membrane pore-forming proteins that respond to environmental stress, such as TolC and OmpX (Aono et al., 1998; Barbosa & Levy, 2000; Dupont et al., 2007). SoxS is a transcriptional activator that positively regulates the expression of over 20 chromosomal genes (Pomposiello et al., 2001; Imlay, 2008). Some of them function to pump out a wide variety of antibiotics, because of an increased synthesis of the AcrAB–ToIC machinery (Aono et al., 1998). Also, the arrest of the outer membrane porin OmpF helps to reduce the rate at which toxic compounds accumulate inside the cell and to protect it from oxidative stress (Cohen et al., 1988; Delihas & Forst, 2001). Hence, the SoxRS regulon system evolved to protect the cell against oxidative stress that specifically arises from redox cycling compounds. In agreement with this explanation, our results directly show that the ompW gene also responds to SoxS, probably functioning as an efflux channel for toxic compounds generated during oxidative stress.

Earlier reports have shown that SoxS preferentially activates certain promoters involved in superoxide resistance and that the soxbox is the critical element in recognition by SoxS (Martin et al., 2000).

![Fig. 4. Transcriptional profiles of the ompW promoter region fused to the cat reporter gene. S. Typhimurium 14028s was independently transformed with fusions of the ompW reporter region. Transformants containing 250, 300, 400 and 500 bp of the ompW promoter region were treated with 4 mM PQ (dark-grey bars) amended at OD_{600} 0.4 and 0.9 or not treated (light-grey bars). CAT specific activity was measured at OD_{600} 1.0 for each culture. The 250 and 500 bp fusions were used to transform S. Typhimurium ΔsoxS and were treated with PQ. Bars represent the mean of three independent experiments and error bars show SD.](image)

![Fig. 5. Specific recognition of the ompW promoter by SoxS. Different PCR fragments (see Fig. 3) of 170 (a), 200 (b), 250 (c) and 300 bp (d) from the ompW promoter region were incubated with increasing concentrations of purified SoxS (0–6.3 μM), and the interaction was resolved by native polyacrylamide gel (6%) electrophoresis. Bands were visualized by ethidium bromide staining.](image)
Binding of purified SoxS to the \textit{ompW} promoter strongly suggests that the PQ- and SoxS-mediated activation of \textit{ompW} is the result of a direct interaction between the promoter and SoxS. A sequence required for PQ-mediated transcriptional induction of \textit{ompW} was mapped by DNase footprinting between positions –54 and –197 upstream of the \textit{ompW} transcriptional start site. This region contained the putative soxbox predicted by \textit{in silico} examination, as well as 25 nt that partially matched a putative SoxS-binding site described by Aono \textit{et al.} (1998) and 17 of 20 nt from the consensus sequence described by Li & Demple (1994) and Martin \textit{et al.} (1999), as seen in Fig. 2.

Examination of the sequence associated with the PQ-inducible \textit{ompW} promoter revealed that SoxS binds with high affinity in a manner characteristic of class I promoters in the forward orientation, such as the \textit{zwf} and \textit{sodA} promoters of \textit{E. coli}. This is in contrast to other promoters implicated in the response to ROS, such as \textit{fpr}, \textit{nfsA} and \textit{yggX}, in which the SoxS binding site is located downstream near to the –35 box (–15 to –30 nt upstream of the –35 sequence) (Martin \textit{et al.}, 1999; 2000; Pomposiello & Demple, 2000; Paterson \textit{et al.}, 2002; Pomposiello \textit{et al.}, 2003; Giró \textit{et al.}, 2006). The \textit{ompW} promoter region contains two recognition sequence elements (underlined) in the soxbox domain (TTTGCATAGGTGATATGTC-AAAATTGAT), in which the GCAY motif is more conserved than the CWA motif (Fig. 2). These elements have been described in protein–DNA interaction studies (Li & Demple, 1994) and by molecular information theory.

**Fig. 6.** DNase I footprinting analysis of the \textit{ompW} promoter region. A 400 bp DNA fragment, corresponding to the region between +30 and –370 of the \textit{ompW} promoter, was obtained by PCR using end-labelled WR primer. An amount equivalent to 25 000 c.p.m. of this labelled DNA was incubated without (0) or with increasing concentrations (0.15–4.8 μM) of purified SoxS. Then, 0.05 U DNase I was added and the mixture was incubated for 1.5 min. The digested DNA was resolved in a sequencing gel, dried and autoradiographed. On the left-hand side, a sequencing reaction of this DNA fragment is shown and the position of the soxbox is indicated. The DNA protected area extends from –54 to –197, as indicated on the right-hand side of the figure.
(Griffith & Wolf, 2001). While the GCAY element would be required for SoxS binding, the CWA element would be necessary for DNA–protein interaction stability (Li & Demple, 1996).

It has not escaped our attention that the pKK232-250 construct, which contains a region between +130 and −120 and does not encompass the whole soxbox predicted in silico, showed the highest CAT reporter activity, and that the corresponding DNA fragment shifted with purified SoxS (Figs 4 and 5). Therefore, our results support the notion that pKK232-250 contains all the cis regulatory elements for optimal SoxS-dependent expression, at least under the conditions studied here. Future research including site-directed mutagenesis should help identify in greater detail the specific nucleotides involved in SoxS biological activity. In this context, when we analysed the sequence downstream of −120, a putative GCAY motif was identified in the −78 position. This element could explain the result obtained in the DNase footprinting experiment (Fig. 6), whereby SoxS protects a region far beyond the soxbox predicted in silico. Hence, the ompW promoter could have two soxbox sequences. Furthermore, Li & Demple (1996) have shown that SoxS can independently bind to multiple sites within individual promoters. In addition, the presence of a soxbox in a promoter, properly aligned and spaced, is enough to convert the gene, not normally a member of the regulon, to one that responds to SoxS in vivo and in vitro (Fawcett & Wolf, 1995).

Accordingly, porins appear not only to contribute to the interaction of a bacterium with its host (Meyer et al., 1998) but also to participate in the uptake of nutrients (Klebba, 2002) and in the influx of ROS, produced both by bacterial metabolism (Yankovskaya et al., 2003) and in response to the oxidative stress generated by the host during pathogenic interaction (van der Straaten et al., 2004).

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