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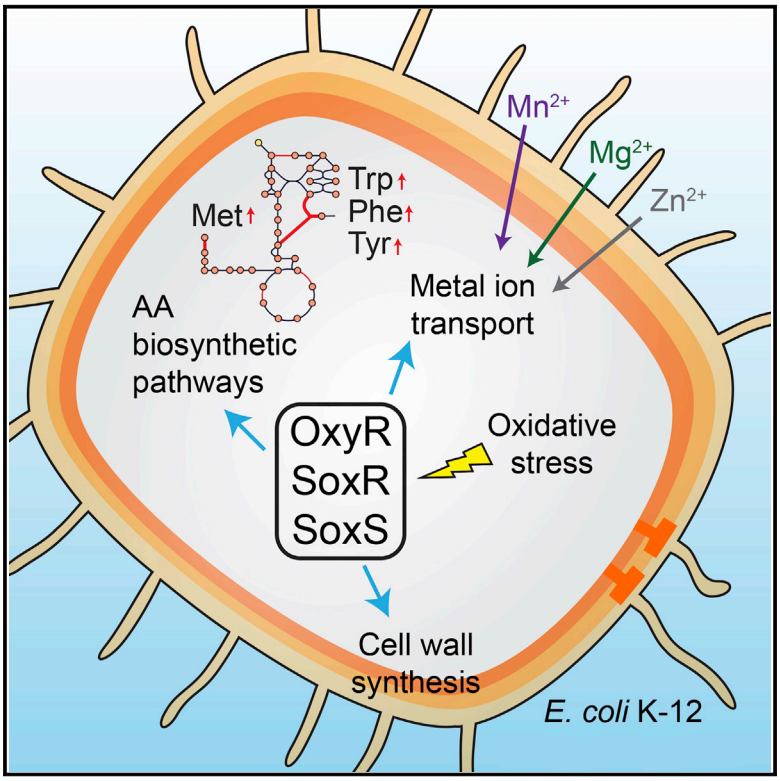
Graphical Abstract



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In Brief

Seo et al. reconstruct OxyR, SoxR, and SoxS transcriptional regulatory networks under oxidative stress in *E. coli* and expand their roles to include direct activation of amino acid biosynthesis, cell wall synthesis, and divalent metal ion transport. These processes are independently regulated by TFs specific to oxidative stress.

Highlights

- Genome-wide reconstruction of OxyR, SoxR, and SoxS regulons in *E. coli*
- 68 genes in 51 TUs belong to these regulons, and 48 genes are directly regulated
- Regulatory roles include direct activation of amino acid biosynthesis
- Roles also include cell wall synthesis and divalent metal ion transport

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Genome-wide Reconstruction of OxyR and SoxRS Transcriptional Regulatory Networks under Oxidative Stress in *Escherichia coli* K-12 MG1655

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SUMMARY

Three transcription factors (TFs), OxyR, SoxR, and SoxS, play a critical role in transcriptional regulation of the defense system for oxidative stress in bacteria. However, their full genome-wide regulatory potential is unknown. Here, we perform a genome-scale reconstruction of the OxyR, SoxR, and SoxS regulons in *Escherichia coli* K-12 MG1655. Integrative data analysis reveals that a total of 68 genes in 51 transcription units (TUs) belong to these regulons. Among them, 48 genes showed more than 2-fold changes in expression level under single-TF-knockout conditions. This reconstruction expands the genome-wide roles of these factors to include direct activation of genes related to amino acid biosynthesis (methionine and aromatic amino acids), cell wall synthesis (lipid A biosynthesis and peptidoglycan growth), and divalent metal ion transport (Mn^{2+}, Zn^{2+}, and Mg^{2+}). Investigating the co-regulation of these genes with other stress-response TFs reveals that they are independently regulated by stress-specific TFs.

INTRODUCTION

Living cells encounter reactive oxygen species (ROS), which are unavoidable in an oxygen-rich environment (Dixon and Stockwell, 2014; Imlay, 2013; Pomposiello and Demple, 2002). These ROS are toxic due to their potential ability to damage oxidizable moieties (for example, in DNA, proteins, and lipids). Superoxide radical ($\cdot O_2^-$) and hydrogen peroxide (H_2O_2) can release iron from the Fe-S-cluster-containing proteins and generate the highly reactive hydroxyl radical ($\cdot OH$) in the intracellular environment (Cabiscol et al., 2000; Cooke et al., 2003). As a primary defense mechanism, prokaryotes have developed antioxidant defense systems through scavenging enzymes, such as superoxide dismutase (SOD), peroxiredoxin, and catalase, to protect cells from ROS damage (Cabiscol et al., 2000; Lushchak, 2001). However, under stressful conditions, these enzymes may be insufficient to protect cells from endogenous ROS. When environmental changes elevate ROS uptake or intracellular formation rates, basal defense systems might become insufficient.

In most gram-negative bacteria, including *Escherichia coli*, two regulatory defense systems are induced under oxidative stress conditions: the OxyR system (Zheng et al., 1998), which responds to hydrogen peroxide, and the SoxR and SoxS systems (Nunoshiba et al., 1992), which respond to redox-active compounds. Several genes regulated by RpoS are also responsible for the oxidative stress response, and indeed, *rpoS* mutants are more susceptible to the lethal effects of exogenous H_2O_2 (Chiang and Schellhorn, 2012). The general roles of these three transcription factors (TFs) and their association with RpoS in detoxification of oxidative stressors have been extensively investigated using mostly in vitro DNA-binding experiments, related mutational analysis, and comparative transcriptomics (Altuvia et al., 1994; Anjem et al., 2009; Fuentes et al., 2001; Haagmans and van der Woude, 2000; Hidalgo et al., 1995; Koh et al., 1996; Koh and Roe, 1996; Kullik et al., 1995; Lee et al., 2009; Li and Demple, 1994; Martin and Rosner, 2002, 2011; Nakayama et al., 2013; Nunoshiba et al., 1993; Partridge et al., 2007; Pomposiello et al., 2001; Ritz et al., 2000; Rungrassamee et al., 2008; Seth et al., 2012; Wenk et al., 2012; Zheng et al., 1999, 2001a, 2001b). However, much less is known about in vivo events including OxyR, SoxR, and SoxS binding at the genome scale and the regulatory networks that they comprise, even in model organisms such as *E. coli* K-12 MG1655. Thus, a complete reconstruction of their transcriptional regulatory networks (TRNs) in response to oxidative stress is expected to reveal a more detailed understanding of their regulations at a genome scale and provide a chance to investigate the inter-relationships between other regulons, particularly those that are related to stress-response TFs. Furthermore, a systems-level understanding of the OxyR, SoxR, and SoxS regulatory networks might shed light on challenging questions about their functions in coordinating stress responses with other fundamental cellular/metabolic processes.

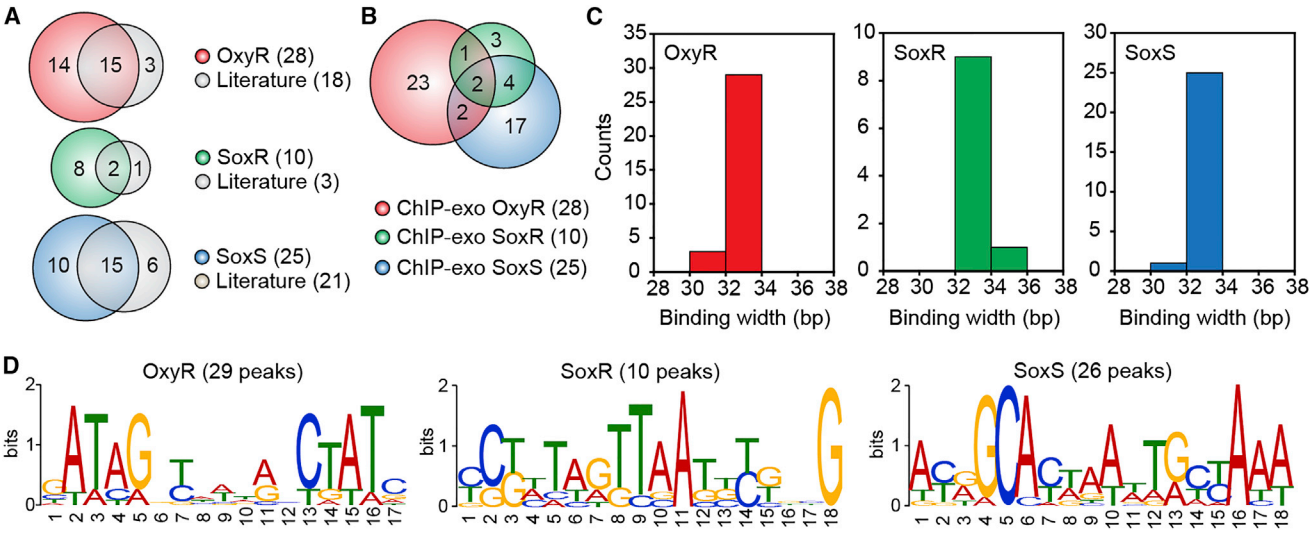

Figure 1. Genome-wide Profiles of OxyR-, SoxR-, and SoxS-Binding Sites

(A) Comparison of genome-wide OxyR-, SoxR-, and SoxS-binding sites obtained from this study (ChIP-exo) under oxidative stress (paraquat treatment) with the known binding sites from the literature.

(B) Overlaps between those binding sites.

(C) Distribution of binding widths for each site. The average binding widths for OxyR, SoxR, and SoxS are 33 ± 0.6, 33.3 ± 0.9, and 32.9 ± 0.2 (bp), respectively.

(D) Sequence motif logo represents the DNA binding motifs of each transcription factor.

See also Figure S1 and Tables S1 and S2.

In this study, we apply a systems biology approach to decode the OxyR, SoxR, and SoxS regulatory networks under oxidative stress (paraquat treatment). We generate genome-scale datasets from chromatin immunoprecipitation with lambda exonuclease digestion followed by high-throughput sequencing (ChIP-exo) for OxyR, SoxR, and SoxS and from strand-specific massively parallel cDNA sequencing (RNA-seq). Those datasets are then integrated to reconstruct the OxyR, SoxR, and SoxS regulons. We first examine TF-binding sites in the *E. coli* K-12 MG1655 genome. We then measure transcription levels of genes in wild-type and knockout mutants of each TF at the genome scale to identify causal relationships. A combination of topological and functional analyses of these regulons provides a comprehensive view of the coordinated genome-wide regulatory roles of these TFs in complex cellular responses under oxidative stress.

RESULTS AND DISCUSSION

Genome-wide Binding Profiles of OxyR, SoxR, and SoxS

Over the last two decades, 18, 3, and 21 binding sites have been identified for OxyR, SoxR, and SoxS, respectively, in *E. coli* by in vitro DNA-binding experiments, mutational analysis, and microarray-based comparative transcriptomics (Altuvia et al., 1994; Anjem et al., 2009; Fuentes et al., 2001; Haagmans and van der Woude, 2000; Hidalgo et al., 1995; Koh et al., 1996; Koh and Roe, 1996; Kullik et al., 1995; Lee et al., 2009; Li and Demple, 1994; Martin and Rosner, 2002, 2011; Nakayama et al., 2013; Nunoshiba et al., 1993; Partridge et al., 2007; Pomposiello et al., 2001; Ritz et al., 2000; Rungrassamee et al., 2008; Seth et al., 2012; Wenk et al., 2012; Zheng et al., 1999, 2001a, 2001b). However, direct measurements of genome-wide in vivo TF binding are not available for *E. coli* K-12 MG1655. Therefore, we first employed a recently developed ChIP-exo method to determine in vivo binding profiles of these three TFs in *E. coli* K-12 MG1655 under paraquat (PQ) treatment. We selected PQ for oxidative stress because it is known to activate both the OxyR and SoxRS systems in *E. coli* (Blanchard et al., 2007; Gu and Imlay, 2011). We used a previously reported 8-myc epitope-tagging approach (Seo et al., 2014) to perform ChIP-exo experiments, and this approach did not compromise the ability of *E. coli* cells to respond to oxidative stress (Figure S1A). From ChIP-exo experiments, we identified 28, 10, and 25 reproducible binding sites for OxyR, SoxR, and SoxS, respectively, under oxidative stress (Table S1). We also detected 76% (32 of 42) of OxyR-, SoxR-, and SoxS-binding sites reported from in vitro experiments (Figure 1A; Table S2). It is unclear why 10 binding sites that were previously identified were not detected from the dataset obtained here. However, there are precedent cases where in vivo ChIP studies were not able to detect a certain number of TF-binding sites that were reported to be detected by in vitro methods (Cho et al., 2008a, 2008b; Federowicz et al., 2014; Myers et al., 2013; Seo et al., 2014). The possible explanation is that other regulators may block bindings of OxyR and SoxRS by competing with them for the same or overlapping binding sites under the particular conditions used here. For instance, the *aceBAK* operon is under a complicated transcriptional regulation by multiple TFs including Fur, Crp, and IclR, and their binding sites overlap with each other (Zhang et al., 2005). Most of the binding sites (43 out of 52) were exclusively occupied by only one TF among those three TFs, leaving 9 binding sites bound by multiple oxidative stress-response TFs

(Figure 1B). The small overlap between these three regulons suggests that these TFs have regulatory networks that are rather independent of each other (Blanchard et al., 2007; Pomposiello and Demple, 2002; Zheng et al., 2001b). Collectively, we significantly expanded the current knowledge of OxyR-, SoxR-, and SoxS-binding information on the genome.

The average widths of the binding footprints of each TF were similar (33 ± 0.6, 33.3 ± 0.9, and 32.9 ± 0.2 bp for OxyR, SoxR, and SoxS, respectively; Figure 1C). All the binding sites were located within regulatory regions (i.e., upstream of promoters, at promoters, and 5′ proximal to coding regions) (Table S1). Except for a few cases where Sigma 38 (RpoS) was involved, most of the promoters were Sigma 70 (RpoD) dependent (Table S1). These results show a strong preference for OxyR-, SoxR-, and SoxS-binding site locations within the noncoding intergenic regions with relatively fixed binding widths.

To identify DNA sequence motifs of these OxyR-, SoxR-, and SoxS-binding sites, we used the MEME tool from the MEME software suite (Bailey et al., 2009) with the genomic sequences of binding sites. The identified sequence motif of OxyR from 29 binding peaks (5′-GATAGBYHWDRVCTATC-3′) was consistent with the previously characterized OxyR-binding site (5′-GAT AGGTTnAACCTATC-3′) (Figure 1D) (Keseler et al., 2011; Salgado et al., 2013). The motif search of SoxR from 10 binding peaks yielded a sequence motif (5′-YCKHTWRKTAAKBYKBBG) that resembled the previously reported motif (5′-CCTCAAGT TAACTTGAGG-3′) (Keseler et al., 2011; Salgado et al., 2013). Likewise, our motif analysis of SoxS from 26 binding peaks showed a sequence motif (5′-AYRGCAYWAWWTRYYAAW-3′) that is similar to the previously reported motif (5′-aTGGCA CaaaaagctAAAca-3′) (Keseler et al., 2011; Salgado et al., 2013). As genome-wide TF-binding data from ChIP experiments can be a source of information for inferring the relative in vivo DNA-binding affinity of TFs (Bailey and Machanick, 2012; Seo et al., 2014), we calculated the signal-to-noise (S/N) ratios of the OxyR-, SoxR-, and SoxS-binding peaks and used them as a proxy of the in vivo binding intensity of each binding site (Table S1). As a result, the more the binding sequence differed from each consensus motif, the lower the in vivo relative binding affinity became (R^2 = 0.53 for OxyR, 0.77 for SoxR, and 0.53 for SoxS; Figure S1B). Based on the coverage of the known binding sites in our data and the agreement of their motifs, we conclude that the OxyR-, SoxR-, and SoxS-binding sites identified here are bona fide binding sites.

Genome-wide Reconstruction of the OxyR, SoxR, and SoxS Regulons

Before this study was conducted, a total of 51 genes in 38 transcription units (TUs) (OxyR, 26 genes in 18 TUs; SoxR, 3 genes in 3 TUs; and SoxS, 28 genes in 21 TUs) had been characterized as members of OxyR, SoxR, and SoxS regulons (Altuvia et al., 1994; Anjem et al., 2009; Blanchard et al., 2007; Fuentes et al., 2001; Haagmans and van der Woude, 2000; Hidalgo et al., 1995; Koh et al., 1996; Koh and Roe, 1996; Kullik et al., 1995; Lee et al., 2009; Li and Demple, 1994; Martin and Rosner, 2002, 2011; Nakayama et al., 2013; Nunoshiba et al., 1993; Partridge et al., 2007; Pomposiello et al., 2001; Pomposiello and Demple, 2002; Ritz et al., 2000; Rui et al., 2010; Rungrassamee et al., 2008; Seth et al., 2012; Wenk et al., 2012; Zheng et al., 1999, 2001a, 2001b). The ChIP-exo datasets of this study expand the size of the OxyR, SoxR, and SoxS regulons to 68 target genes in 51 TUs (OxyR, 38 genes in 28 TUs; SoxR, 11 genes in 10 TUs; and SoxS, 34 genes in 25 TUs; several genes are co-regulated) (Table S1). It should be noted that 38 target genes in 29 TUs were previously identified (Table S1). Almost half of these target genes (47%, or 32 of 68) were metabolic genes, and the majority of them (96%, or 65 of 68) were non-essential genes (Figure S1C). In addition, 15% (10 of 68) were regulatory genes, such as *ybaO* and *micF*, that were either TFs or small RNAs (sRNAs).

To determine the causal relationship between the binding of TFs and changes in RNA transcript levels of their regulon genes, we compared transcript levels of wild-type and each deletion mutant (Δ*oxyR*, Δ*soxR*, and Δ*soxS*), both grown under stress condition with PQ treatment. Overall, a total of 227 genes were differentially expressed in at least one mutant ($\log_2$ fold change $\geq$ 1 and false discovery rate [FDR] $\leq$ 0.01) (Figure 2A). Only 7 of 227 were differentially expressed in all mutants, which explains the consequence of the small overlap between target genes of three TFs. (Figure 2A; Tables S3, S4, and S5). Combining the genome-wide TF-binding maps with TF-dependent transcriptomes enabled us to determine the causal relationship between the binding of each TF and the changes in transcript levels of the corresponding genes under oxidative stress (Figures 2B and 2C). Among 68 target genes identified from ChIP-exo analysis, we determined that of 48 genes in 36 TUs (OxyR, 26 genes in 17 TUs; SoxR, 8 genes in 7 TUs; and SoxS, 19 genes in 16 TUs) were directly regulated by OxyR, SoxR, and SoxS under oxidative stress (Figure 3; Table S1). Of the 20 genes that were not differentially expressed by single-TF-knockout experiments, 11 genes (*dsbG*, *uof-fur*, *yhjA*, *oxyS*, *fhuF*, *acrAB*, *micF*, *oxyR*, and *soxR*) were previously known as regulon members of OxyR and/or SoxRS (Table S1). It is possible that other co-regulating TFs may partially take over the regulatory role on each gene under our experimental condition when the relevant TF is missing. For example, OxyR and SoxS co-activate the expression of *uof-fur*, and thus, knockout of either one may only result in a marginal change in the expression level (i.e., less than 2-fold). Indeed, *uof-fur* showed a 1.3- and 1.9-fold change in transcript levels in Δ*oxyR* and Δ*soxS* mutants compared to wild-type (FDR $\leq$ 0.01). Likewise, four other genes (*dsbG*, *fhuF*, *acrA*, and *acrB*) also showed a more than 1.5-fold but less than 2-fold change in transcript levels. Unfortunately, autoregulation of *oxyR* and *soxR* could not be detected in our knockout experiments. In addition, *clpS* and *yhjB*, OxyR targets identified in this study, showed a 1.8- and 1.5-fold change in transcript levels, respectively, when OxyR was deleted. Although we were not able to detect changes in expression levels of the remaining 10 genes, we detected reproducible binding peaks and motifs at the promoter regions of these genes. We decided to compare the characteristics of two different gene sets for further analyses, which include either all 68 genes or the 48 genes showing causal relationships, respectively.

The calculation of the relative distance from binding centers of these TFs to transcription start sites (TSSs) (Keseler et al., 2011; Kim et al., 2012) showed that OxyR, SoxR, and SoxS preferably

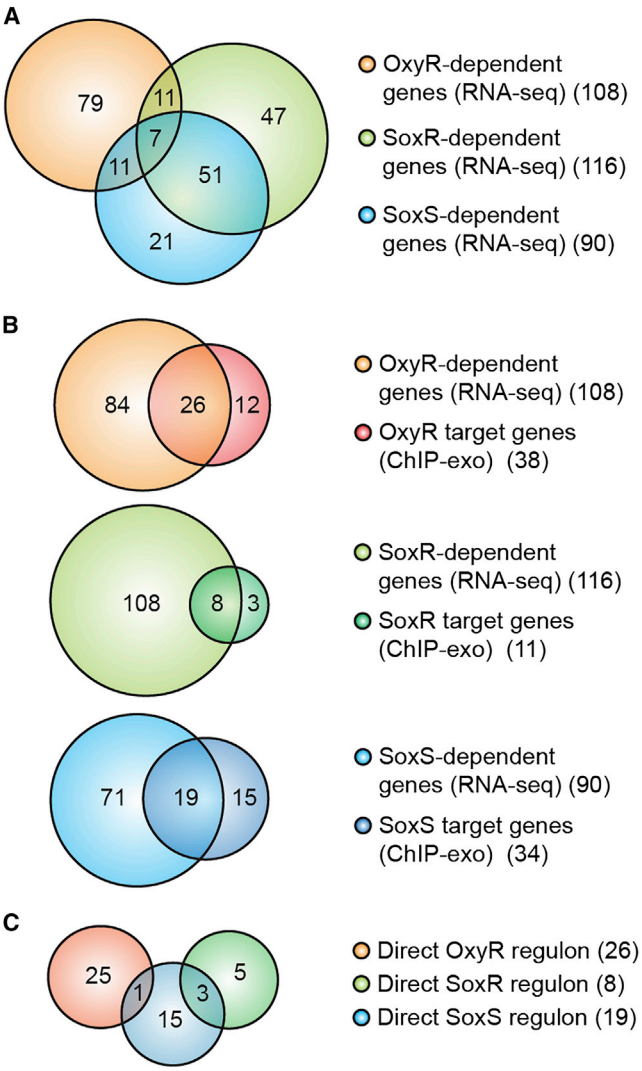

Figure 2. Regulatory Causation in OxyR, SoxR, and SoxS Regulons

(A) Overlaps between OxyR-, SoxR-, and SoxS-dependent transcriptomes under oxidative stress.

(B) Causal relationships between direct associations of transcription factors and changes in transcript levels of genes.

(C) Overlaps between direct regulons of each TF.

See also Figure S2 and Tables S3, S4, and S5.

bind to the sites either further upstream or downstream of the promoter region when they act as activators (Figure S2A). This result suggests that they may prevent binding of other repressors or directly activate transcription through additional mechanisms, such as recruiting RNAP to activate transcription. In cases where these TFs act as repressors, their bindings are localized around the RNAP-binding regions so that the repression of transcription can be achieved by exclusion of RNAP binding due to the occupancy of these TFs at this region (Figure S2B). The characteristics of binding to the promoter of genes that did not show a regulatory response upon the deletion of a TF were similar to those of activation or repression cases (Figure S2C), indicating that these bindings would regulate target genes, while this was not observed under single-TF-knockout conditions.

It is known that SoxR induces *soxS* expression, and SoxS, in turn, activates the transcription of genes involved in the defense against oxidative stress. Thus, the known scope of transcriptional regulation by SoxR was quite limited to *fumC*, *soxS*, and itself (Fuentes et al., 2001; Hidalgo et al., 1995). Surprisingly, we found that SoxR also directly activated several other genes other than *soxS*, such as *lpxC*, *aroF*, *sodA*, and *mgtA* (Table S1). We also discovered that SoxS directly activated one interesting putative TF, *ybaO*, which has been reported to restore cysteine-responsive regulation in a *Salmonella enterica* Δ*cutR* mutant (Oguri et al., 2012). Since cysteine is reduced under oxidative stress due to its Fenton reaction (Park and Imlay, 2003), this result suggests that YbaO may act as an additional regulator of cysteine metabolism in response to oxidative stress once SoxS activates its expression.

Expanded Genome-wide Regulatory Roles of OxyR, SoxR, and SoxS

Next, we sought to study the functions of the gene products in these three regulons. As stated before, we set up two gene groups: one including all 68 target genes and the other including 48 genes with direct regulation under the single-TF-knockout condition. We classified them based on clusters of orthologous groups (COG) protein database and found that two categories, post-translational modification and inorganic ion transport and metabolism, were statistically overrepresented in both groups (hypergeometric test p value $<3 \times 10^{-3}$) (Figure S3). This result is consistent with the properties of cellular responses to oxidative stress for detoxification (*sodA*, *katG*, and *ahpCF*) and damage repair (*dps*, *nfo*, *trxC*, *grxA*, and *sufABCDSE*) (Figures 4A and 4B) (Altuvia et al., 1994; Kullik et al., 1995; Ritz et al., 2000; Zheng et al., 2001a).

It is known that oxidative stress induces metabolic responses such as the activation of *zwf*, encoding glucose 6-phosphate-1-dehydrogenase, by SoxS to increase NADPH pools and promote antioxidant defense by mediating the reduction of thioredoxins and glutaredoxins (Henard et al., 2010; Prinz et al., 1997). We also observed activation of *zwf* by SoxS, while regulation of other genes that are responsible for NADPH production, such as *gnd*, *icdA*, and *maeB*, were not detected. Furthermore, a previous study claimed that SoxS directly activates enzymes in the tricarboxylic acid cycle, such as *acnA* (encoding aconitase A), which is known to be more stable and resistant to oxidation in vivo than the isozyme AcnB (Varghese et al., 2003). SoxS also activates *fumC* (encoding fumarase) with association of RpoS, which is less sensitive to oxidative damage because it is an iron-independent enzyme unlike its other two isozymes (FumA and FumB) (Liochev and Fridovich, 1993). Similarly, we found that OxyR and SoxR directly activated the expression of several other metabolic genes in amino acid biosynthesis pathways to overcome inactivation of reactions possibly by oxidative stress. Two enzymes, 2-dehydro-3-deoxyphosphoheptonate aldolase (DAHP synthase, encoded by *aroF*) and homocysteine transmethylase (encoded by *metE*) required for aromatic amino acids and methionine synthesis, were directly activated by SoxR and OxyR, respectively (Figure 4C). Since these enzymes are reported to be vulnerable to oxidative stress (Hondorp and Matthews,

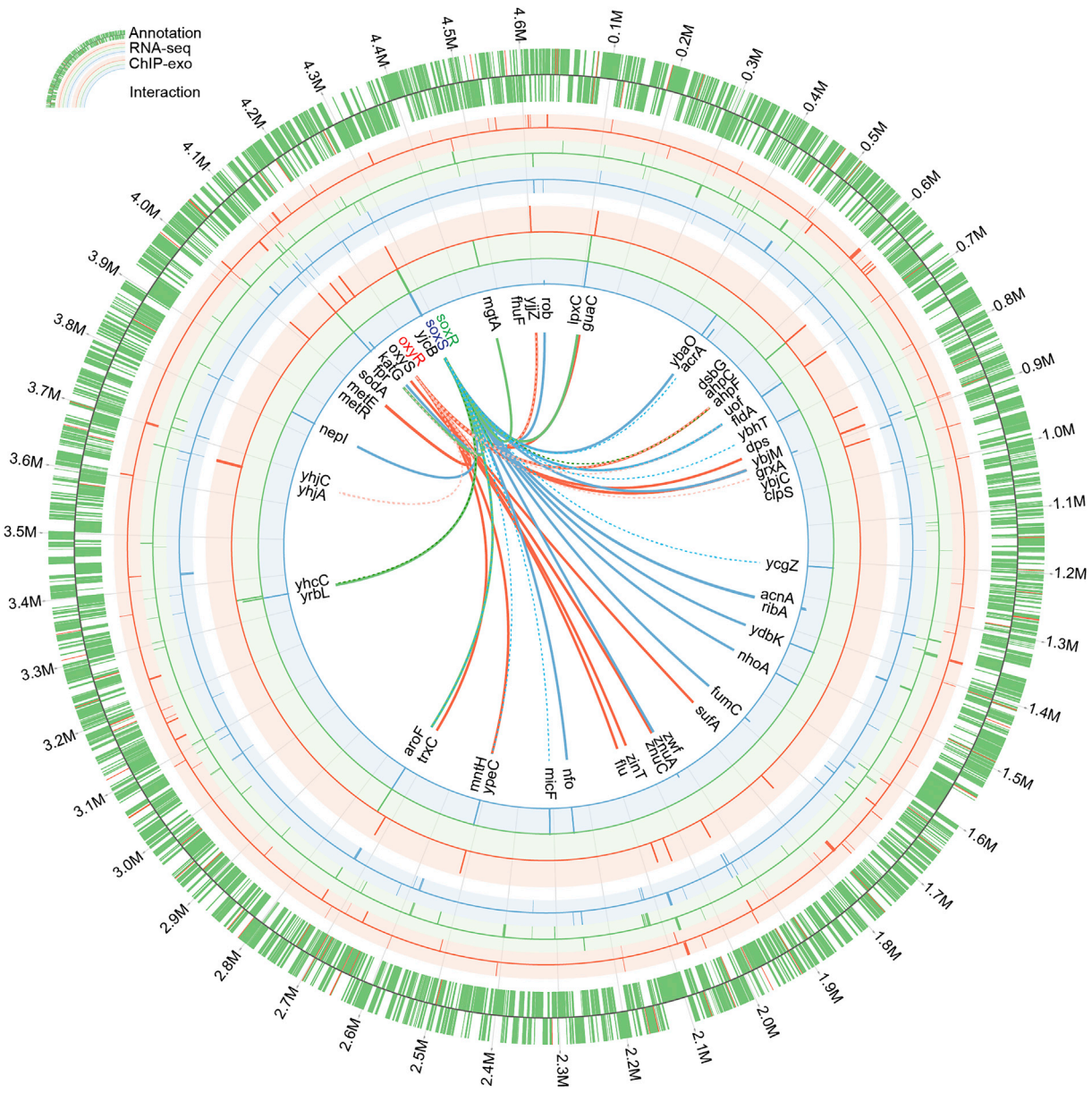

Figure 3. Genome-wide Map of OxyR, SoxR, and SoxS Regulons

Genomic map of OxyR-, SoxR-, and SoxS-binding profiles overlaid with transcriptional response to TF knockout under oxidative stress. Red, green, and blue denote OxyR, SoxR, and SoxS, respectively. Dotted lines indicate the genes with TF bindings but without changes in expression level upon single TF knockout. Only the first gene of the operon was shown. The figure was made using Circos version 0.67. See also Figure S2 and Table S1.

2004; Sobota et al., 2014), it appears that *E. coli* may try to increase expression of *aroF* and *metE* to overcome the shortage of essential amino acids. The increased metabolic flux toward the aromatic amino acid biosynthetic pathway under superoxide stress from the previous study (Rui et al., 2010) supports this observation. In addition, the activation of *metR* by OxyR can also lead to the activation of another transmethylase encoded by *metH* under the presence of cobalamin cofactor. This series of activations of key enzymes along the pathways would allow cells to achieve metabolic robustness against oxidative stress.

These TFs also regulated genes related to cell wall synthesis. SoxR and SoxS directly activated *lpxC* that encodes UDP-3-O-acyl-N-acetylglucosamine deacetylase that catalyzes the second step of lipid A biosynthesis (Figure 4D). Although *lpxC* has been hypothesized to be a regulon member based on a comparative transcriptome study in *E. coli* (Pomposiello and Demple, 2002), we provide strong evidence of both TF bindings and causal relationships for this hypothesis. Furthermore, OxyR also directly activated *mepM*, encoding murein DD-endopeptidase, which is known to have the RpoS-dependent promoter (Figure 4D). Its

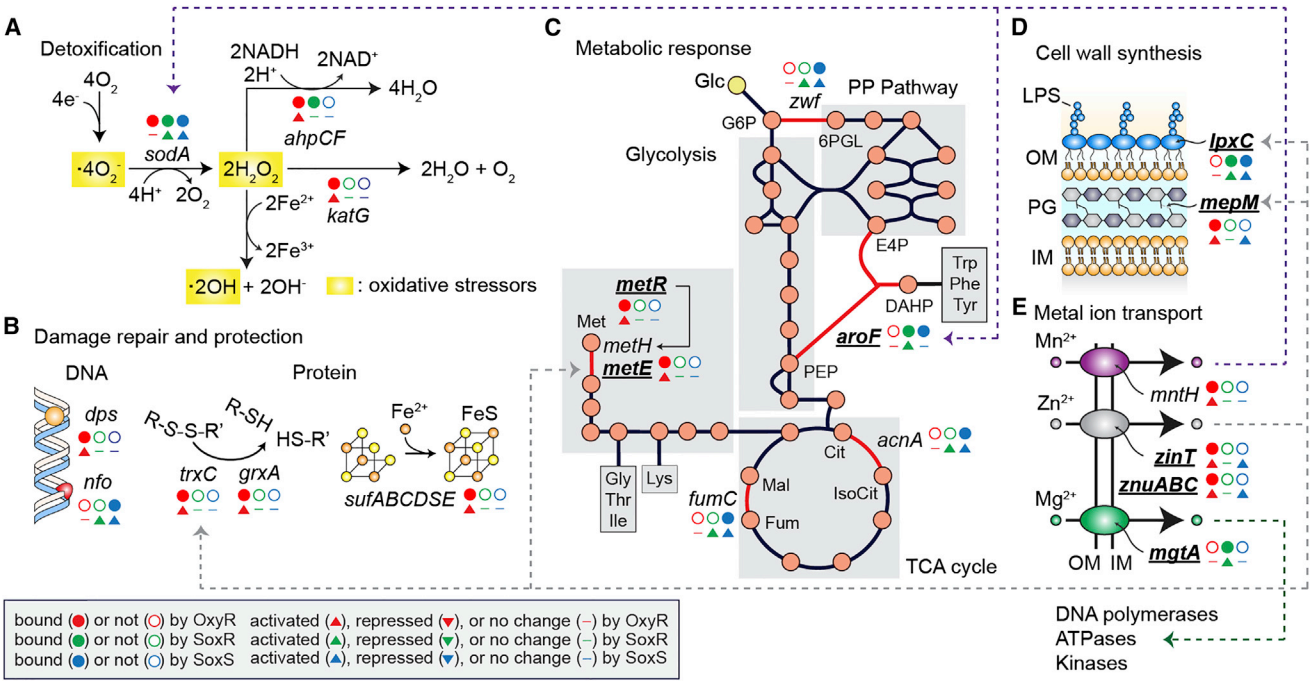

Figure 4. Functional Delineation of OxyR, SoxR, and SoxS Regulons

(A and B) We confirmed that OxyR, SoxR, and SoxS directly regulate genes associated with (A) detoxification and (B) DNA protection/damage repair and protein damage repair.

(C–E) The additional regulatory functions of OxyR, SoxR, and SoxS identified in this study. These TFs directly regulate genes associated with (C) metabolic robustness (methionine and aromatic amino acid biosynthesis), (D) cell wall synthesis (lipid A biosynthesis and peptidoglycan growth), and (E) divalent metal ion transport (Mn^{2+}, Zn^{2+}, and Mg^{2+}).

Bold and underlined characters indicate OxyR, SoxR, and SoxS regulons identified in this study. Closed and open circles represent whether target genes are bound by the TFs or not. Triangles, inverted triangles, and dashes indicate whether the expression levels of target genes are activated, repressed, or not changed upon TF knockout, respectively. Dotted lines indicate requirements of divalent metal ions for functions of enzymes. Red, OxyR; green, SoxR; blue, SoxS. LPS, lipopolysaccharide; PG, peptidoglycan; OM, outer membrane; IM, inner membrane. See also Figures S3 and S4 and Table S1.

primary role is to cleave the crosslinks between D-alanine (ala) and *meso*-diaminopimelic acid (mDAP) in order to facilitate growth of peptidoglycan (PG). This endopeptidase is required for a new PG incorporation (Singh et al., 2012). Thus, the activation of these two genes, *lpxC* and *mepM*, would result in structural changes or increased outer membrane thickness, possibly to withstand the oxidative stress coming from the environment.

Previously, OxyR was known to activate *mntH* encoding manganese (Mn^{2+}) transporter (Anjem et al., 2009), while both OxyR and SoxS have been known to activate *fur* encoding ferric uptake regulator (Fur) to repress Fe^{2+} uptake under oxidative stress (Zheng et al., 1999). Surprisingly, we found that OxyR and SoxR also directly activated transporters of other divalent transition metal ions such as Zn^{2+} (*zinT*/*znuABC*) and Mg^{2+} (*mgtA*) (Figure 4E). Among enzymes that are activated in response to oxidative stress, SodA and AroF require Mn^{2+}, and TrxC, LpxC, MepM, and MetE require Zn^{2+} to function properly (Keseler et al., 2011). Based on these requirements, these divalent metal ions become crucial to overcome oxidative stress with these enzymes. It is unclear why MgtA, Mg^{2+} transporter, is activated by SoxR under oxidative stress. However, magnesium is generally coordinated in protein complexes such as the active sites of DNA polymerases, ATPases, and kinases.

In order to further examine the physiological roles of the OxyR, SoxR, and SoxS regulon members identified in this study, we chose eight genes (*nepI*, *lpxC*, *yjcB*, *mepM, yrbL, ybaO*, *nhoA*, and *yjjZ*) that were either activated or showed no causal relationship under single-TF-knockout conditions. We also chose *dps* because it was implicated in oxidative stress tolerance in *E. coli* O157:H7 (Choi et al., 2000). Susceptibility assays of single-knockout strains of these genes revealed that five of them were more sensitive to oxidative stress (Figure S4). They include genes related to DNA damage repair (*dps*), cell wall synthesis (*lpxC* and *mepM*), NAD^+ homeostasis (*nepI*) (Park et al., 2013), and unknown function (*yjcB*). This result indicates that OxyR, SoxR, and SoxS contribute to the orchestration of global oxidative stress responses in *E. coli* beyond detoxification and damage repair.

Interaction between OxyR, SoxR, and SoxS Regulons and with Other Stress-Response TFs

After a genome-wide functional analysis of these regulons, we further classified their target genes into 13 detailed functional categories with their annotated functions in order to investigate the inter-relationship between regulons under oxidative stress and other stress conditions (Figure 5). Within an oxidative stress

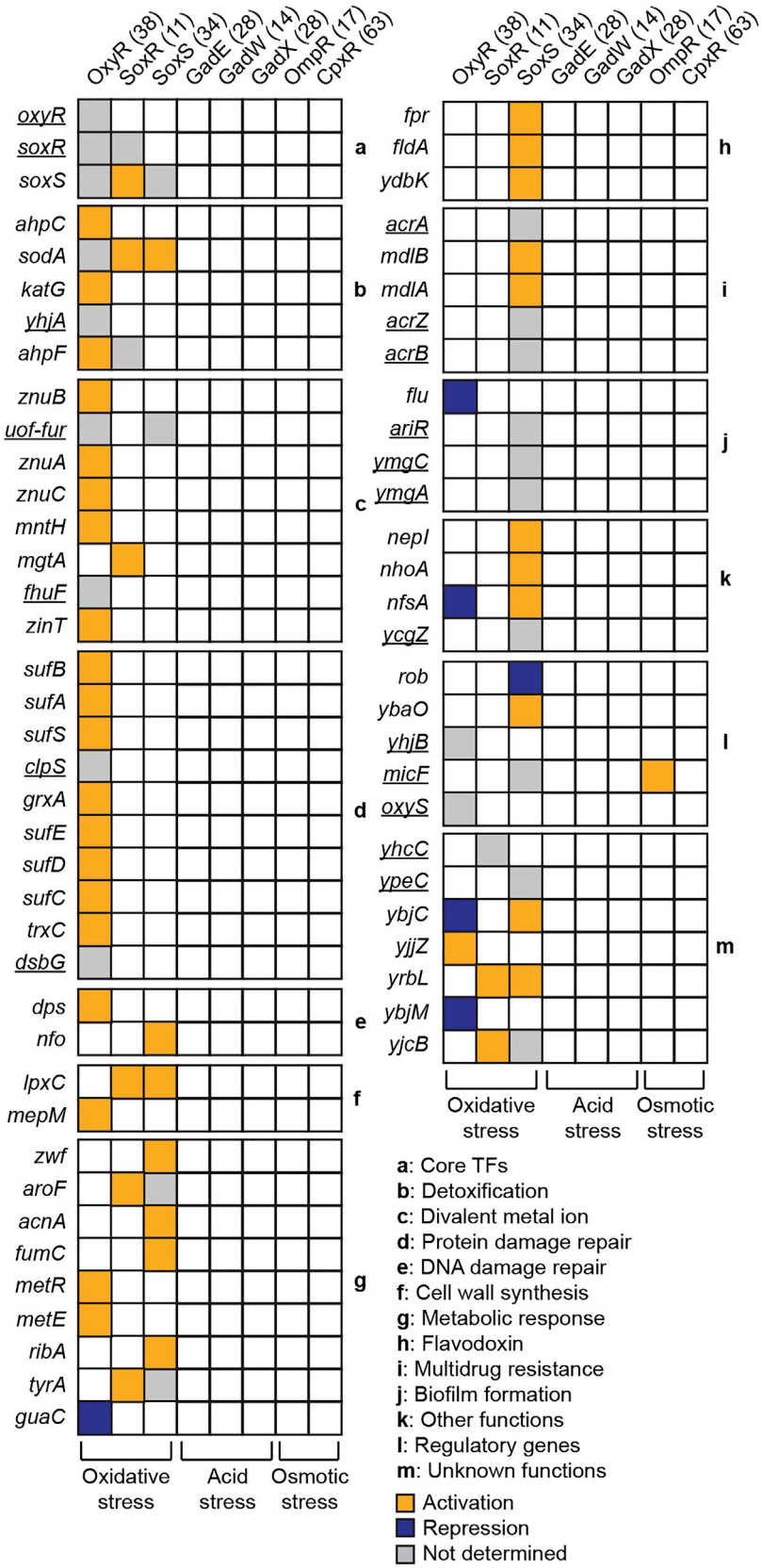

Figure 5. OxyR, SoxR, and SoxS Regulon Members and Their Interactions with Other TFs

Integration of the OxyR, SoxR, and SoxS regulon information (68 target genes) with other publicly available regulon information of stress-responsive TFs for acidic (GadE, GadW, and GadX) and osmotic (OmpR and CpxR) stresses. The 68 target genes were categorized into 13 groups (a–m) based on their functional annotations. The underlined 20 genes showed less than 2-fold changes in expression level under relevant TF-knockout conditions. The amber and dark blue indicate activation and repression of transcription by direct association of each TF, respectively. The gray indicates the transcription regulation could not been determined though the direct association with a TF. See also Figure S5 and Tables S6 and S7.

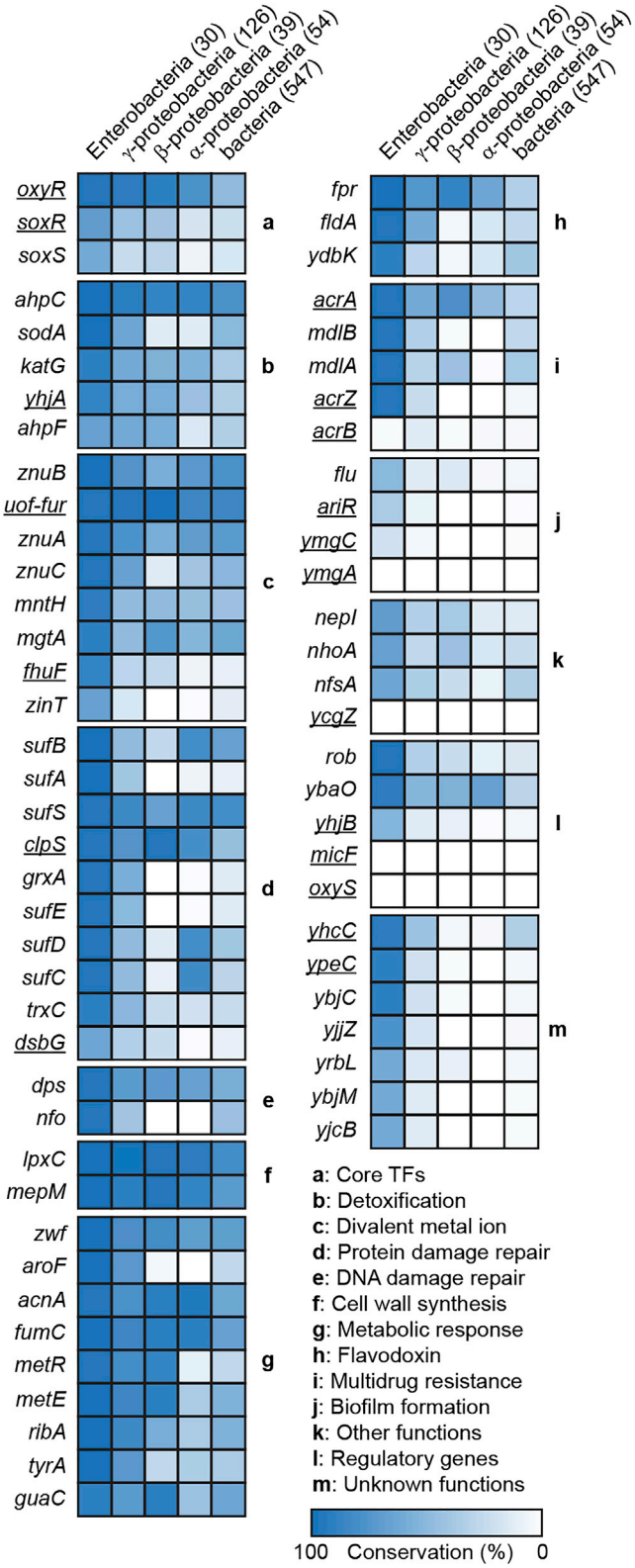

Figure 6. Evolutionary Perspective on OxyR, SoxR, and SoxS Regulons

Conservation levels of the OxyR, SoxR, and SoxS regulon genes across enterobacteria; γ-, β-, and α-proteobacteria; and bacteria are illustrated by the ortholog calculation. The genes were divided into 13 groups by according to function as in Figure 5. The underlined 20 genes showed a less than 2-fold change in expression level under relevant TF-knockout conditions.

response, we clearly observed that 80% of target genes (55 of 68) were regulated by only one of three TFs. We found that OxyR is more responsible for regulating detoxification (b), divalent metal ion metabolism (c), and protein damage repair (d) processes. On the other hand, SoxS regulates several processes in flavodoxin (h), multidrug resistance (i), and biofilm formation (j). Other processes such as DNA damage repair (e), cell wall synthesis (f), and metabolic response (g) were regulated by more than one TF.

We further integrated the information from the OxyR, SoxR, and SoxS regulons with other publicly available regulon data of TFs related to acid (GadE, GadW, and GadX) and osmotic (OmpR and CpxR) stresses based on the strong evidence from previously published data and RegulonDB (Salgado et al., 2013; Seo et al., 2015) (Figure 5). Surprisingly, 98% (67 of 68) of OxyR, SoxR, and SoxS regulon members, all but *micF*, were not regulated by any of these five TFs. When we only considered 48 genes that showed direct regulation under single-TF-knockout conditions, they were not regulated by other stress-related TFs. We extended our analysis to include the entire list of TFs. 46% (31 of 68) of the regulon members (Figure S5A) and 44% (21 of 48) of genes that showed direct regulation (Figure S5B) still have no known regulatory action by any other TF (Tables S6 and S7). Unlike the characteristics of the sigma factor networks that appear to provide the cell with regulatory redundancies (Cho et al., 2014), these stress-response TFs regulated transcription of target genes in response to each stress with minimal overlap between regulons.

Evolutionary Aspects of the OxyR, SoxR, and SoxS Regulons

Given the fact that oxidative stress response is crucial for the survival of bacteria under aerobic growth conditions (Dixon and Stockwell, 2014), we speculated that the presence of genes required for stress response might be relatively conserved across species. Thus, we further investigated how *E. coli* OxyR, SoxR, SoxS, and their regulons have been conserved within a bacterial kingdom by comparing 30 Enterobacteriaceae, 126 γ-proteobacteria, 39 β-proteobacteria, 54 α-proteobacteria, and 547 bacteria. As expected, members of the OxyR regulon required for stress response tend to be widespread (a, b, c, and d in Figure 6) compared to that of SoxS regulons (h, i, j, and k in Figure 6). This result is in agreement with the fact that SoxS is found only in enterobacteria (Dietrich et al., 2008). Recently, it has been reported that *lpxC* belongs to the OxyR regulon in *Pseudomonas* (Wei et al., 2012), but our study showed that it belongs to both SoxR and SoxS in *E. coli*. In other bacteria, the preserved SoxR tends to control a distinct set of genes involved in redox-active antibiotic production (Dietrich et al., 2008). Based on these facts, it might be the case that OxyR (or another functional ortholog, PerR, when OxyR is not present; Chiang and Schellhorn, 2012) takes over the roles of SoxR and SoxS.

In summary, we have described an integrative analysis of various data types from cutting-edge genome-scale experimental methods and how this systems approach enabled us to comprehensively understand the complex roles of the OxyR, SoxR, and SoxS regulatory networks in response to oxidative stress in *E. coli*. The expanded scope of oxidative stress response accomplished by OxyR, SoxR, and SoxS are related to other fundamental cellular processes such as amino acid biosynthesis (methionine and aromatic amino acids), cell wall synthesis (lipid A biosynthesis and peptidoglycan growth), and divalent metal ion transport (Mn^{2+}, Zn^{2+}, and Mg^{2+}). Understanding ROS responses in microbes has important implications for antibiotic treatment (Brynildsen et al., 2013; Dwyer et al., 2014; Kohanski et al., 2007). Specifically, the prediction via a genome-scale model of *E. coli* metabolism with ROS production potentiated the killing by oxidants and antibiotics (Brynildsen et al., 2013). The inclusion of the expanded regulatory networks revealed in this study under oxidative stress into the current model would increase the predictive capability of the model for targeting newly identified resistant strains (Brynildsen et al., 2013; O'Brien et al., 2013). Collectively, our approach could be easily extended to other TFs in *E. coli* or other species for elucidation of regulons and the complex networks that they comprise.

EXPERIMENTAL PROCEDURES

Bacterial Strains, Media, and Growth Conditions

All strains used are *E. coli* K-12 MG1655 and its derivatives. The *E. coli* strains harboring OxyR-8myc, SoxR-8myc, and SoxS-8myc were generated by a λ-Red-mediated site-specific recombination system targeting C-terminal region of each gene (Cho et al., 2006). Deletion mutants (Δ*oxyR*, Δ*soxR*, and Δ*soxS*) were also constructed by a λ-Red-mediated site-specific recombination system (Datta et al., 2006). Glycerol stocks of *E. coli* strains were inoculated into fresh 70 ml M9 minimal medium (47.8 mM Na_2HPO_4, 22 mM KH_2PO_4, 8.6 mM NaCl, 18.7 mM NH_4Cl, 2 mM $MgSO_4$, and 0.1 mM $CaCl_2$) supplemented with 0.2% (w/v) glucose in a 500-ml flask and cultured overnight at 37°C at 250 rpm. To create oxidative stress, the overnight cultures were inoculated to an optical density 600 (OD_{600}) = 0.01 into the fresh 70 ml of M9 minimal medium in a 500-ml flask supplemented with 250 μM paraquat (PQ) at OD_{600} = 0.3 ± 0.03 and incubated for 20 min with stirring.

ChIP-Exo

To identify OxyR-, SoxR-, and SoxS-binding maps in vivo, we isolated the DNA bound to each TF from formaldehyde cross-linked *E. coli* cells by chromatin immunoprecipitation (ChIP) with the antibodies that specifically recognizes myc tag (9E10, Santa Cruz Biotechnology) and Dynabeads Pan Mouse IgG magnetic beads (Invitrogen) followed by stringent washing steps (Cho et al., 2008a). ChIP materials (chromatin-beads) were used to perform on-bead enzymatic reactions of the ChIP-exo method (Rhee and Pugh, 2012) with modifications as shown in our previous study (Seo et al., 2014, 2015) to construct sequencing libraries. Prepared DNA libraries were sequenced using MiSeq (Illumina) in accordance with the manufacturer's instructions. ChIP-exo experiments were performed in biological duplicate. Sequence reads generated from ChIP-exo were mapped onto the reference genome (NC_000913.2) using bowtie (Langmead et al., 2009) with default options to generate SAM output files. MACE program (https://code.google.com/p/chip-exo/) (Wang et al., 2014) was used to write in-house script to define peak candidates from biological duplicates with sequence depth normalization. To reduce false-positive peaks, peaks with signal-to-noise (S/N) ratio less than 1.0 and same signal with mock immunoprecipitation were removed as in our previous study (Seo et al., 2014, 2015).

RNA-Seq Expression Profiling

Total RNA including sRNAs was isolated using the cells treated with RNAprotect Bacteria Reagent (QIAGEN) followed by purification using QIAGEN RNeasy Plus Mini Kit (QIAGEN) in accordance with the manufacturer's instructions. Paired-end, strand-specific RNA-seq was performed using the dUTP method (Levin et al., 2010) with the modifications as shown in our previous study (Seo et al., 2014; Seo et al., 2015) to build the sequencing library. The samples were sequenced using MiSeq (Illumina) in accordance with the manufacturer's instructions. RNA-seq experiments were performed in biological duplicate. Sequence reads generated from RNA-seq were mapped onto the reference genome (NC_000913.2) using bowtie (Langmead et al., 2009) with the maximum insert size of 1,000 bp and two maximum mismatches after trimming 3 bp at 3′ ends. These files were then used for Cufflinks (http://cufflinks.cbcb.umd.edu/) (Trapnell et al., 2010) and Cuffdiff to calculate fragments per kilobase of exon per million fragments (FPKM) and differential expression, with default options and library type of dUTP RNA-seq. From cuffdiff output, genes with differential expression with log2 fold change $\geq$1.0 and an FDR $\leq$ 0.01 were considered as differentially expressed genes.

Motif Search and Analysis

The OxyR-, SoxR-, and SoxS-binding motif analyses were completed using the MEME tool from the MEME software suite with default settings (Bailey et al., 2009).

COG Functional Enrichment

The OxyR, SoxR, and SoxS regulons were categorized according to their annotated clusters of orthologous groups (COG) category. Functional enrichment of COG categories was determined by performing one-tailed Fisher's exact test (hypergeometric test), and a p value < 0.05 was considered significant. Two categories (post-translational modification, chaperones and inorganic ion transport and metabolism) were statistically significant.

Conservation Analysis of OxyR, SoxR, and SoxS Regulons

Gene annotation of strains and species were obtained from the SEED server (http://theseed.org), and ortholog calculation to *E. coli* K-12 MG1655 was also performed on the RAST (Rapid Annotation using Subsystem Technology) server (Aziz et al., 2008). Conservation levels of *oxyR*, *soxR*, *soxS*, and genes that belong to OxyR, SoxR, and SoxS regulons were calculated from orthologs retained from the RAST output.

Susceptibility Assays under Oxidative Stress

Susceptibility assays followed the procedures of a previous study with modification of usage of growth media (Minakami and Fridovich, 1990). Cells grown overnight in M9 minimal media (pH 7.0) were diluted into fresh M9 media and cultured to OD_{600} = 0.4. These cell cultures were inoculated into TSY media (30 g tryptic soy broth and 5 g yeast extract per liter, adjusted to pH 7.0) supplemented with 5 mM paraquat. The initial cell density inoculated for oxidative stress was between 5×10^5 and 2×10^6 colony-forming units (CFUs) per milliliter. The cultures were then incubated at 37°C with 250 rpm shaking, and samples were collected after 2 hr and 4 hr. Aliquots were serially diluted, and triplicates were plated onto TSY agar plates. Colonies were counted after 24 hr. Percent survival was calculated as follows: [(CFU/ml at time 2 hr or 4 hr)/(CFU/ml at time 0)] × 100. The results presented are averages of triplicate experiments and include the SDs.

ACCESSION NUMBERS

All raw data for ChIP-exo and RNA-seq are available under the accession number GEO: GSE65712.

SUPPLEMENTAL INFORMATION

Supplemental Information includes five figures and seven tables and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2015.07.043.

AUTHOR CONTRIBUTIONS

S.W.S., D.K., and B.O.P. designed research. S.W.S and D.K. constructed myc-tagged and knockout strains. S.W.S. performed ChIP-exo and RNA-seq. R.S. ran MiSeq. S.W.S., D.K., and B.O.P. analyzed data. S.W.S., D.K., and B.O.P. wrote and edited the manuscript.

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