Coordination of frontline defense mechanisms under severe oxidative stress

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Complexity of cellular response to oxidative stress (OS) stems from its wide-ranging damage to nucleic acids, proteins, carbohydrates, and lipids. We have constructed a systems model of OS response (OSR) for Halobacterium salinarum NRC-1 in an attempt to understand the architecture of its regulatory network that coordinates this complex response. This has revealed a multi-tiered OS-management program to transcriptionally coordinate three peroxidase/catalase enzymes, two superoxide dismutases, production of rhodopsins, carotenoids and gas vesicles, metal trafficking, and various other aspects of metabolism. Through experimental validation of interactions within the OSR regulatory network, we show that despite their inability to directly sense reactive oxygen species, general transcription factors have an important function in coordinating this response. Remarkably, a significant fraction of this OSR was accurately recapitulated by a model that was earlier constructed from cellular responses to diverse environmental perturbations—this constitutes the general stress response component. Notwithstanding this observation, comparison of the two models has identified the coordination of frontline defense and repair systems by regulatory mechanisms that are triggered uniquely by severe OS and not by other environmental stressors, including sub-inhibitory levels of redox-active metals, extreme changes in oxygen tension, and a sub-lethal dose of γ rays.

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Introduction

Reactive oxygen species (ROS), such as hydrogen peroxide (H2O2), superoxide (O2−), and hydroxyl (OH−) radicals are normal by-products of aerobic metabolism. Evolutionarily conserved mechanisms including detoxification enzymes (peroxidase/catalase and superoxide dismutase (SOD)) and free radical scavengers manage this endogenous production of ROS. Oxidative stress (OS) is a condition reached when certain environmental stressors or genetic defects cause the production of ROS to exceed the management capacity. The damage to diverse cellular components including DNA, proteins, lipids, and carbohydrates resulting from OS (Imlay, 2003; Apel and Hirt, 2004; Perrone et al, 2008) is recognized as an important player in many diseases and in the aging process (Finkel, 2005).

A great deal of information exists on specific detoxification enzymes, ion scavengers, and their associated regulators. For instance, in Escherichia coli, it is known that the oxyR regulon is induced by higher levels of H2O2, whereas the soxRS regulon is activated by exposure to redox-cycling agents (Zheng and Storz, 2000; Green and Paget, 2004; Imlay, 2008). Activation of many of these regulators by metals, radiation, and starvation conditions shows how diverse environmental factors (EFs) can directly or indirectly increase production of ROS. Not surprisingly, the regulation of OS response (OSR) is tied to circuits that manage diverse processes including metal trafficking, nutrient transport, and general aspects of metabolism. The function of metal trafficking is especially important given the function of transition metals, iron in particular, in the production of highly reactive OH− through the Fenton reaction. It is clear from all of these earlier studies that a systems approach will be necessary to fully appreciate the highly interconnected nature of the regulatory networks that manage OSR (Temple et al, 2005).
Given that many archaea thrive in extreme environments associated with elevated production of ROS, it is intriguing to consider these organisms as models for characterizing global regulatory networks that efficiently manage extreme OS during normal metabolism (Joshi and Dennis, 1993; Kawakami et al., 2004; Limauro et al., 2008). Furthermore, although archaeal transcriptional regulation is mediated by regulators of bacterial ancestry, many of the known bacterial OSR regulators are absent in this domain of life (Coulson et al., 2007). Here, we have applied a systems approach to characterize the OSR of an archaeal model organism, *Halobacterium salinarum* NRC-1. This halobacterium grows aerobically at 4.3 M salt concentration in which it routinely faces cycles of desiccation and rehydration, and increased ultraviolet radiation—both of which can increase the production of ROS (Farr and Kogoma, 1991; Oliver et al., 2001). We have reconstructed the physiological adjustments associated with management of excessive OS through the analysis of global dynamic transcriptional changes elicited by constant exposure to growth sub-inhibitory and sub-lethal levels of H2O2 and paraquat (PQ—a redox-cycling drug that produces O2; Hassan and Fridovich, 1979) as well as during subsequent recovery from these stresses. We have integrated all of these data into a unified model for OSR to these ROS to discover conditional functional links between protective mechanisms and normal aspects of metabolism. Subsequent phenotypic analysis of gene deletion strains has verified the conditional detoxification functions of three putative peroxidase/catalase enzymes, two SODs, and the protective function of rhodopsins under increased levels of H2O2 and PQ. Similarly, we have also validated ROS scavenging by carotenoids and flotation by gas vesicles as secondary mechanisms that may minimize OS. Although it has been known that OS is a component of diverse environmental stress conditions, we quantitatively show for the first time that much of the transcriptional responses induced by the two treatments could indeed have been predicted using a model constructed from the analysis of transcriptional responses to changes in other EFs (UV and γ-radiation, light, oxygen, and six metals). However, using specific examples, we also reveal the specific components of the OSR that are triggered only under severe OS. Notably, this model of OSR gives a unified perspective of the interconnections among all of these generalized and OS-specific regulatory mechanisms.

### Results and discussion

We present the results and discussion in six sections (A–F) starting with (A) characterization of growth and survival characteristics of *H. salinarum* NRC-1 under increasing doses of H2O2 and PQ, (B) analysis of global dynamic transcriptional responses to selected sub-inhibitory and sub-lethal doses of the two stressors, (C) inference of a globally integrated model of conditionally co-regulated genes and their sub-circuits, (D) experimental validation of important hypotheses regarding primary and secondary defense mechanisms and their functional relationships, (E) statistical evaluation of generalized and specific aspects of OS management, and (F) summary of the integrated program for OS management.

### Distinct differences in phenotypic responses to H2O2 and PQ

We first characterized survival and growth characteristics of *H. salinarum* NRC-1 with increasing doses of H2O2 and O2. Although peroxide stress was generated by directly adding H2O2 to a desired concentration (titration doses for survival studies: 0–40 mM; growth studies: 0–7 mM), O2 radicals were generated indirectly during metabolism of exogenously added PQ (titration doses for survival studies: 0–10 mM; growth studies: 0–0.5 mM) (Hassan and Fridovich, 1979) (Figure 1). Interestingly, the cells withstood exogenously added H2O2 stress up to a concentration of ~30 mM beyond which their survival dropped precipitously. This sharp transition was also observed at the level of growth wherein the cells grew normally in up to 5–6 mM H2O2 and a smaller increase thereafter resulted in complete growth inhibition. This indicates that in the absence of earlier conditioning to low doses of OS (Cabiscol et al., 2000; Brioukhanov et al., 2006; Limauro et al., 2006), there exists a threshold to the cellular capacity to detoxify H2O2 and that even traces of peroxide beyond this threshold can result in devastating oxidative damage to cellular components. This phenotypic behavior under H2O2 stress is in stark contrast to the gradual loss in survival and growth observed under increasing O2 stress. The growth and survival assays aided in the selection of appropriate concentrations of H2O2 and PQ for subsequent transcriptomic analysis. The enormous amount of the two stress agents required to achieve sub-lethal effects is a testament to the remarkable capacity of this organism to efficiently eliminate and keep intracellular concentration of ROS within physiologically acceptable limits.

### Global transcriptional analysis reveals shared and specific responses to peroxide and O2

We sought to model OSR at a systems scale by characterizing OS-management mechanisms and unraveling the gene regulatory programs that coordinate their operation with other aspects of metabolism. We approached this problem by charting dynamic global transcriptional changes for up to 4 h during and after exposure to sub-lethal concentrations of each PQ (4 mM) and H2O2 (25 mM) that resulted in 20–30% loss of survival (Figure 2; Supplementary Figures S1 and S2; Supplementary Tables S1–S3). Further, we also characterized transcriptional responses during nearly 6 h of acclimation to a growth sub-inhibitory dose of PQ (0.25 mM) (this was not possible for peroxide stress owing to the sharp transition in growth with small concentration increases of H2O2) (Supplementary Figure S3; Supplementary Table S4). Together with control experiments and replicates, this represented nearly 300 high-density microarray experiments. We summarize discoveries from integrated analyses of data from these experiments (Materials and methods) (Supplementary Figure S1). Exposure to both ROS resulted in differential regulation of a larger proportion of genes over time involving up to 50% of all genes at steady state. In contrast, transcriptional changes became somewhat subdued over time during recovery from both OS conditions (Supplementary Figure S1). Nearly half of
all differentially regulated genes (563 genes) were shared by the two stress responses—perhaps reflecting a generalized OS component (Supplementary Table S1). Notably, differential regulation of an equivalent fraction of genes (H2O2: 366 genes; PQ: 536 genes) was unique to each response. Some unique aspects of responses to the two ROS and between responses to sub-lethal and sub-inhibitory doses of PQ are noted in the Supplementary information (Figure 2; Supplementary Figures S2 and S3). By simultaneously analyzing putative functional assignments (Bonneau et al., 2004) and transcriptional changes, we have reconstructed physiological consequences of long-term exposures to and recovery from excessive H2O2 and O2 stress (Figure 2; Supplementary Figures S2–S3; Supplementary Tables S2–S4). We provide a summary of this analysis with emphasis on OS-related functions; observations pertaining to adjustments in general aspects of physiology including ribosome biogenesis, oxidative phosphorylation, and carbohydrate and nucleotide metabolism during the OSR are summarized in the Supplementary information.

ROS scavenging and detoxification

Many of the primary OS-management systems including detoxification by SODs, peroxidase/catalase, and peroxiredoxins, and radical scavenging by antioxidants, and reduction of radicals by redoxins (Storz and Imlay, 1999; Grant, 2001; Zheng et al., 2001; Toledano et al., 2007; Imlay, 2008) were upregulated by both treatments (Figure 2O, P and S; Supplementary Table S2). Notably, in addition to upregulation of the classic peroxidase/catalase [VNG6294G (perA)—PF00141], we observed transcriptional induction of at least two additional enzymes of related function—VNG0798H, a putative dye-decolorizing peroxidase (PF04261, COG2837) (Sugano et al., 2007; Scheibner et al., 2008) and VNG0018H, whose predicted structure matches that of catalase [PDB: 1cf9A2; (Mate et al., 1999)]. VNG1190G (sod1) and peroxiredoxins [VNG1197G (bcp), VNG2311H] were upregulated to a higher degree with PQ than with H2O2, suggesting that different regulatory circuits are responsible for managing ROS generated by the two treatments (Brioukhanov et al., 2006; Limauro et al., 2006, 2008). Upregulation of a number of other processes was also consistent with their functions in relation to OS including carotenoid synthesis [VNG1458G (crtB1), VNG1755G (crtI2)] and glycerol metabolism [VNG6277G (ugpB), VNG6279G (ugpA), VNG1967G (glpK), VNG6270G (gldA)] (Pahlman et al., 2001) (Figure 2A, B, and N).

Repair of oxidative damage

Although DNA repair mechanisms were in general regulated in a similar manner by both agents (Figure 2T; Supplementary Table S2), PQ treatment uniquely upregulated certain repair and recombination genes including VNG2473G (radA1), ssDNA binding (VNG1255G and VNG2160G), and DNA
Figure 2  Temporal transcriptome changes in H. salinarum NRC-1 treated with sub-lethal doses of H$_2$O$_2$ (25 mM) or PQ (4 mM). Relative changes in transcription of all genes were measured immediately before and up to 160 min after adding 25 mM H$_2$O$_2$ or 4 mM PQ. The mRNA log$_{10}$ ratios were normalized to the first condition (control) and data were filtered (log$_{10}$ ratio $>$ 0.05, $\lambda_1$=15 in at least one condition; see Materials and methods for details). I. Differentially regulated genes are grouped into different functional groups (A–U) (see Supplementary Table S2 for details). II. Transcriptional profiles that distinguished OSRs to H$_2$O$_2$ and PQ (Groups V and W) were identified using the significance analysis of microarrays (SAM) algorithm (Tusher et al, 2001).
helicase [VNG1406G (rhl)]. Although it is clear from upregulation of proteases [VNG0205H, VNG0354C, VNG2096G (cttB), VNG0226G (htrA), VNG1951G (sub), VNG2323H, VNG6201G (hsps), VNG6361G (hnps)] that protein degradation was upregulated by both treatments (Figure 2U), it was striking that unlike the upregulation of ribosomal genes for replacing damaged proteins (Thorpe et al, 2004) under H2O2 stress, these genes were downregulated with PQ—even after the stress was taken away. We speculate that the sub-lethal dose of PQ (4 mM) was more damaging to proteins relative to H2O2 (25 mM), despite their comparable phenotypic effects (20–30% loss in survival). This is not surprising as PQ primarily generates O2− ions, which are known to be particularly deleterious to proteins as they increase the production of highly reactive OH− radicals through the Fenton reaction by releasing free Fe2+ from solvent-exposed 4Fe-4S cluster of labile dehydratases (Imlay, 2003). As expected, lowering the dose of PQ to sub-inhibitory levels induced transcription of both proteases and ribosome biogenesis (Supplementary Figure S3).

Regulation of Fe–S metabolism
Most transcriptional changes induced by both treatments were over-represented for Fe-dependent and Fe–S-containing enzymes (Supplementary Figure S4; Supplementary Tables S5 and S6). For instance, a simple analysis of Fe–S cluster-associated motif-containing proteins shows that six of eight (P=0.1) putative electron transfer system proteins (CX3CX2CX2C) and seven of nine (P=0.08) putative enzymes requiring S-adenosylmethionine (SAM) (CX2CX2C) were differentially expressed under OS (Supplementary Tables S5 and S6). This is not surprising as Fe–S clusters are used as cofactors for many redox proteins, radical SAM enzymes, and RNA polymerase (Boyd et al, 2009), and are important for electron transfer reactions and gene regulation as they sense changes in Fe, O2, and O2− (Fontecave, 2006; Outten, 2007) (Figures 2, 3; Supplementary Tables S2 and S3). It has been proposed that conversion of Fe2+ to Fe3+ could trigger three types of responses: a Fe-deficiency such as response to bring in more Fe into the cell; an effort to repair or re-synthesize proteins with damaged Fe–S clusters and S-containing amino acids; and a protective response to sequester Fe in a non-reactive form (Varghese et al, 2007; Imlay, 2008). Consistent with this, cellular effort to scavenge and sequester Fe, and simultaneously repair both damaged Fe–S clusters and S-containing amino acids was reflected in the upregulation of genes encoding Fe-scavengers: putative siderophore biosynthesis [VNG6213G (iucB), VNG6212G (iucA), VNG6214G (hxyA), VNG6216G (iucC)] (Imlay, 2006); iron sequestration [ferritin [VNG2244G (dpsA)] (Andrews et al, 2003; Reindel et al, 2006; Shukla, 2006)]; S uptake [VNG1592G (cysT2)]; assembly of Fe–S clusters [VNG0524G (yurY), VNG0525C, VNG0527C] (Takahashi and Tokumoto, 2002; Loiseau et al, 2003); biosynthesis of cysteine and methionine [VNG0796G (cys)], VNG2420G (meta), VNG2421G (hal), and VNG1301G (cysK)]; and repair of oxidized cysteines and methionines [VNG1180G (msrA)] (Stadtman et al, 2003; Stadtman, 2006; Metayer et al, 2008) (Figure 2; Supplementary Table S2).

An integrated predictive model for OS management
Data presented here and earlier (Causton et al, 2001) have clearly shown that OSR is a global phenomenon impacting a wide array of cellular processes encoded by at least 50% of all genes (Figure 2; Supplementary Figure S2 and S3; Supplementary Table S1). We have taken a step toward construction of a predictive model for global transcriptional coordination of the OSR. Briefly, using the cMonkey algorithm (Reiss et al, 2006), we integrated transcriptome changes, gene functional associations (phylogenetic profiles, chromosomal proximity, operon organization, metabolic networks, etc.), and de novo discovered conserved cis-regulatory motifs to identify subsets of genes that are conditionally co-regulated by OS (biclusters). Subsequently, we constructed an environment and gene regulatory influence network for OS (EGRINOS) by performing linear regression and model selection using the Inferelator algorithm (Bonneau et al, 2006, 2007). Analysis of regulatory influences of TFs and the two OS agents on each bicluster within this model has recapitulated earlier known regulatory phenomena for managing OS and revealed new putative regulatory links among diverse cellular processes.

Discovery of co-regulated genes reveals new regulatory links among different OS components
Altogether, 1165 genes were grouped into 100 biclusters using the cMonkey algorithm (Supplementary Table S7). Using two examples, we show how this integrated the analysis of genes, environmental conditions, functional associations, and shared cis-regulatory motifs within individual biclusters has discovered numerous regulons and provided evidence for conditional co-regulation of diverse functions during OSR (Figure 3).

Example 1: Analysis of bicluster #84 (bc84) has provided evidence for the potential transcriptional co-regulation of sod1 with several redoxins, a thioredoxin reductase, bacterioferri- tin, cysteine biosynthesis/repair enzymes, molecular chaperones, and five genes with no matches to protein sequence or signature of characterized function. Though it has been shown that sod1 and cysteine repair systems are part of the SoxRS regulon and Fe scavenging is part of the OxyR regulon (Imlay, 2008), global analysis has shown the two regulons to be overlapping (Farr and Kogoma, 1991; Zheng et al, 2001). Similarly, the gene composition of bc84 suggests that this regulon is analogous to a functional hybrid of the SoxRS and OxyR systems in E. coli. However, there are no orthologs for either of these regulators in H. salinarum NRC-1 (Coulson et al, 2007). Subsequent regulatory network inference with Inferelator has helped identify potential regulator(s) that mediate this control (see below). Notably, co-expression of the 23 genes was especially prominent in PQ-treated cells. Although this is consistent with the O2−-specific function of some of these genes (e.g. sod1), it also suggests similar ROS-specific functions for some of the other genes.

Example 2: In bc12, we have discovered potential co-regulation of genes encoding siderophore biosynthesis, ferrichrome transport, a thioredoxin reductase, and eight earlier unknown function proteins. The regulatory link
between siderophore biosynthesis and ferrichrome transport is not surprising given their shared functions in managing intracellular Fe pool (Andrews et al., 2003), and their conditional co-regulation during an OSR, especially H₂O₂ stress, in E. coli and Bacillus subtilis (Imlay, 2008). However, evidence for co-regulation of this process with a chromosome partitioning gene \[\text{VNG6153G} (\text{sojC2})\], thioredoxin reductase \[\text{VNG2301G} (\text{trxB3})\], and eight genes of unassigned functions is new information. A single conserved palindromic motif within the promoters of all genes within this bicluster suggested that this regulon is under the direct control of a single TF (Figure 3).

In comparing the distribution of genes in the two biclusters discussed above, we also note that this integrated analysis has helped hypothesize condition-specific functions for genes of related function [redox homeostasis (e.g. thioredoxin reductase, glutaredoxin, and peroxiredoxins), 12 Fe-management genes, proteolysis, cobalamin biosynthesis, and O₂ metabolism] as well as putative functions for at least three genes (\[\text{VNG1092C}, \text{VNG0248C}, \text{VNG2299H}\]) of earlier unknown

**Figure 3** Discovery of conditionally co-regulated genes reveals novel physiological links. Conditionally co-regulated genes were discovered using the cMonkey algorithm (see text for details). (A, B) Shows two example biclusters: bc84 (23 genes) and bc12 (21 genes), respectively. The plots show mean and variance-normalized transcriptional changes (log₁₀ ratios) for all genes (gray) in each bicluster with an overlaid averaged profile (black). Filled triangles on x axis indicate time course analysis of responses during continued exposure to 25 mM H₂O₂ or 4 mM PQ. The open triangles, on the other hand, indicate time course analysis of recovery from 2 h exposure to 25 mM H₂O₂ or 4 mM PQ. Genes and their putative functions are indicated in the table. Sequence logos for the conserved motifs are shown in lower right of each panel, with numbers indicating nucleotide position and size of letters indicating relative conservation of bases. Genes possessing conserved DNA sequence motifs are marked with squares, triangles, or circles. (B) Lower right: operon-like organizations of nine genes within bicluster 12.
function. Interactive exploration of all biclusters to enable similar discoveries is possible online at http://baliga. systemsbiology.net/drupal/content/egrin-oxidative-stress.

A unified model for global transcriptional coordination of responses to treatments with H$_2$O$_2$ and PQ

OSR regulators are typically associated with redox-active cofactors such as haem, flavins, pyridine nucleotides, Fe–S clusters, and redox-sensitive amino-acid side chains such as cysteiny1 thiolis (Green and Paget, 2004). Altogether, 26 TFs and 17 signal transducers [containing methyl-accepting chemotaxis protein (MCP)-signaling domain] in _H. salinarum_ NRC-1 have matches to motif signatures for these OS-sensing features (Zheng and Storz, 2000; Fomenko and Gladyshev, 2002; Paget and Buttner, 2003; Fontecave, 2006). A significant fraction of these regulators (21 TFs and 13 signal transducers) was differentially regulated in response to H$_2$O$_2$ and/or PQ treatments (Supplementary Table S5). Although the high degree of cross-regulation that we observed might result from chemical inter-conversions among ROS (Liochev and Fridovich, 2007), it is more likely because of regulators that sense a globally affecting parameter such as the overall redox status (Demple, 1997; Zheng and Storz, 2000) or metal ion composition of the cell (Touati, 2000; Moore and Helmann, 2005; Lee and Helmann, 2006). However, even though the trigger (e.g. redox status) for many of these regulators is the same, their mechanism of activation and the genes they regulate are nonetheless different. In _E. coli_, for instance, in response to O$_2$ stress, SoxR gets activated through oxidation of its Fe–S cluster and regulates a specific subset of genes that is distinct from those controlled by OxyR, which is activated through oxidation of cysteine residue(s) under H$_2$O$_2$ stress (Zheng and Storz, 2000; Imlay, 2008). In our study as well, we have observed unique transcriptional responses, such as upregulation of three thioredoxins [VNG1012H, VNG1259G, and VNG2600G] and a DNA repair gene VNG2473G (radA1) by PQ, but not by H$_2$O$_2$, suggesting involvement of several ROS-specific regulatory mechanisms in mediating the global OSR.

We have taken a step toward a systems level model (Figure 4) that collectively exploits these OS-induced gene-expression changes as a function of shared and unique influences of TFs and two ROS. These regulatory influences in EGRIN$_{OS}$ recapitulate the global transcriptional changes observed during an OSR (Supplementary Figure S5). EGRIN$_{OS}$ also provides meaningful insight into the interrelationships among stress-management systems and general aspects of physiology under H$_2$O$_2$ and PQ stress (Figure 4). The architecture of the model reveals that the OSR network has significant overlap with response networks that manage other kinds of stress. For instance, a common set of TFs are predicted to coordinate genes in bc6, which contains the putative dye-decolorizing peroxidase (VNG0798H), with those in bc84 (contains sodI) and bc12 (contains Fe-management proteins) (bc84 and bc12 are discussed in detail above). Likewise, OSR genes within bc26 (a monoxygenase, several thioredoxin reductases, methionine sulfoxide reductase _msrA_, cysteine synthetase _cysK_, and a putative protease) are positively influenced by the same putative TF (ImlD1) that also negatively influences transcription of cobalamin synthesis and ATP synthase genes in bc91. Finally, three biclusters containing genes of diverse redox-reactive functions (bc17—carotenoid biosynthesis; bc33—siderophore biosynthesis; bc71—antioxidant synthesis) are co-regulated by TFs with redox-sensing and metal-binding domains. In the following sections, we show how EGRIN$_{OS}$ can be used as a framework to formulate experimentally testable hypotheses for characterizing general and specific components of the OSR.

The architecture of this statistically inferred network (Figure 4A) can be used to formulate hypotheses regarding combinatorial control of cellular processes during an environmental response, OSR in this case. These hypotheses can be tested by validating physical interactions of TFs with promoters of genes they are predicted to regulate, and by analyzing transcriptional and phenotypic consequences of genetically perturbing TFs in the network. Using a combination of such experimental validations, we have overcome the complexities presented by extensive buffering within such networks because of multiple redundancies for regulation and stress management. We have validated regulatory influences of six TFs (Trh4 and five general transcription factors (GTFs—TFBb, c, d, f, and g) in the OSR network by showing their physical interactions with promoters of target genes, and consequence of deleting some of these TFs on expression of these genes (TFBd and c) and, more importantly, on survival rate (TFBc) under OS. Specifically, we have validated combinatorial control of genes within the same bicluster (bc88) by multiple TFs (Trh4, TFBb, TFbf, and TFBg; Figure 4B; Supplementary Table S9), differential regulation of genes in different biclusters (bc3, bc81, bc72, and bc33) by the same TF (TFBd) (Figure 4C), and diminished capacity to withstand OS on deleting a predicted TF (TFBc) within this network (Figure 4D). The consequence of deleting TFBc on increased susceptibility to OS can now be further investigated in the context of its function in coordinating specific processes as predicted by EGRIN$_{OS}$.

**Phenotypic analysis of mutants reveals a multi-tiered program for OS management**

In addition to validating architecture of EGRIN$_{OS}$, we have also conducted phenotypic analysis of strains with in-frame chromosomal deletions in genes identified to be important in OS management based on their putative functions, their transcriptional changes in response to OS, and the EGRIN$_{os}$ model. Specifically, we investigated the functions of two SODs, three putative peroxidase/catalase genes, carotenoids, rhodopsins, and gas vesicles in OS management (Figures 5 and 6).

Two SODs (SOD1 and SOD2) offer protection against PQ treatment but not against H$_2$O$_2$

According to the EGRINos model, the two SOD genes are not co-regulated even by PQ treatment, which predominantly generates O$_2$ stress in which their function is expected to be most relevant, suggesting distinct functions for the two orthologs. The co-regulation of VNG1190G (sodI) with genes encoding methionine sulfoxide reductase [VNG1180G...
Figure 4  EGRINOS: environment and gene regulatory influence network for oxidative stress. (A) EGRINOS was constructed by applying the Inferelator algorithm to infer predictive regulatory influences of TFs and EFs (H2O2 and PQ) on biclusters of conditionally co-regulated genes discovered using cMonkey. To simplify visualization, TF groups in the EGRINOS network are indicated with a red border on relevant nodes. Circles indicate single TFs or groups of TFs. ‘TF groups’ are averaged expression profiles of their member TFs (see table). Triangles indicate a logical ‘AND’ combination of regulatory influences. A red star indicates that the influence of a TF (red font) within a particular group has been experimentally validated. Corresponding position of the TF in the network is marked with a red star. Rectangles with numbers indicate specific biclusters; width of each box is proportional to number of conditions in that bicluster and height is proportional to number of genes within the same bicluster (bc84). Further, these TFs also bind promoters of genes in other biclusters that they are predicted to regulate. The network is drawn as a composite of P–D interactions and statistically inferred influences by Inferelator. Thickness of edges are proportional to significance of promoter binding in ChIP-chip experiments, whereas the activate (red arrows) and repress (green edges) influences are based on Inferelator predictions. (B) TFBd binds the promoters of at least four genes grouped into as many distinct biclusters. Consistent with its predicted influence a knockout in TFBd results in significantly lower transcript levels of these genes at all stages of growth in batch culture. This example validates the EGRINOS predicted regulation by TFBd and illustrates how a TF coordinates transcription of genes involved in different processes during OSR. (D) Finally, the deletion of TFBc, which is predicted to coordinate amino-acid metabolism and iron metabolism genes, results in increased sensitivity to 4 mM PQ (transcription factor-binding sites for the six TFs (indicated with red stars in panel A) are listed in Supplementary Table S9).
Together with the observation that sod2 was upregulated only by treatment with a sub-lethal dose of PQ (Figure 2; Supplementary Figure S3), this suggested that this ortholog provides marginal protection against excessive O$_2$ stress. However, this does not rule out a more significant function for this enzyme under specialized conditions (Valderas and Hart, 2001). Notably, unlike in other organisms (Carlioz and Touati, 1986), neither of the two SODs, individually or together, provided any protection against H$_2$O$_2$ stress (Figure 5B).

**Figure 5** Phenotypic analyses of mutants under PQ and H$_2$O$_2$ stress. (A) Growth analysis of SOD single and double gene knockout mutants in the presence of 0.125 mM PQ. (B) Survival of mutants after a 2 h treatment with either 4 mM PQ or 25 mM H$_2$O$_2$. (C) Survival of mutants after 2 h treatment with 5, 15, or 25 mM H$_2$O$_2$. *H. salinarum NRC-1 Δura3* is the parent strain that was used as a control in all experiments.

**Figure 6** Secondary protective mechanisms against OS. (A) Endogenous H$_2$O$_2$ levels during aerobic growth of Δura3 and ΔperA strains were quantified using the Amplex Red assay. Although H$_2$O$_2$ levels were consistently low throughout growth of the parent strain, as expected the levels were significantly higher in the peroxidase mutant. However, the unexpected growth of this mutant coincided with drop in H$_2$O$_2$ level, suggesting the presence of an alternate mechanism(s) to detoxify H$_2$O$_2$. (B) Defective gas vesicle biogenesis in the peroxidase mutant. To assay for flotation properties, cells were grown to stationary phase at 37°C and then left undisturbed (i.e. without shaking) at room temperature on the bench top. Although the parent Δura3 strain floated to the surface, the peroxidase/catalase mutant sank to the bottom of the flask. Microscopy analysis confirmed that gas vesicles (observed as bright refractile bodies in the parent strain) are completely missing in the peroxidase/catalase mutant. (C) The function of carotenoids and other pigments was predicted to be important based on the bleached appearance of stationary phase cultures of the double Δsod1/Δsod2 mutant. This was confirmed on measuring and calculating a fourfold increase in the rate of degradation of pigments relative to single sod mutants (Δsod1, Δsod2) and the parent Δura3 strain. Pigments were assayed using UV-VIS spectroscopy.
resulted in poor growth under oxic conditions and hypersensitive 62% reduction in survival, respectively (Figure 5C; Supplementary Figure S6). The onset of growth of the ΔperA strain after a prolonged lag phase coincided with a corresponding drop in intracellular H₂O₂ content from ~6 to <2 µM (levels observed in typical wild-type cell cultures; all measurements of H₂O₂ were normalized to 10⁶ cells) (Figure 6A) (Gonzalez-Flecha and Demple, 1997; Seaver and Imlay, 2001). This suggested the presence of additional mechanism(s) to handle peroxide stress. The EGRINOS model suggested two candidate genes: VNG0798H and VNG0018H. VNG0798H, which has a weak borderline match to a protein family signature for dye-decolorizing peroxidases (PF04261, e-value ~0.02), was upregulated by both H₂O₂ and PQ, and co-expressed with carotenoid biosynthesis enzymes, gas vesicle biogenesis proteins, and a putative heat shock protein (bc85) [http://baliga.systemsbiology.net/drupal/content/egrin-oxidative-stress]. Moreover, VNG0798H shares Inferelator-discovered regulatory influences with sod1 and Fe-management proteins [section ‘A unified model for global transcriptional coordination of responses to treatments with H₂O₂ and PQ’; Figure 4]. Likewise, VNG0018H, whose predicted protein structure (in lieu of absence of a sequence-based match to characterized proteins) matches that of a catalase (PDB: 1cf9A2; Mate et al, 1999) (Bonneau et al, 2004) was upregulated under H₂O₂ stress. Knockouts in these candidate genes validated their functions in providing partial protection against H₂O₂ stress (Figure 5C). Although deletion of perA resulted in complete loss of viability at 25 mM H₂O₂, deletion of VNG0798H and VNG0018H resulted in 99.9 and 62% reduction in survival, respectively (Figure 5C; Supplementary Figure S6). Furthermore, knockouts in perA and VNG0018H also resulted in 54 and 67% loss in survival, respectively, when challenged with 4 mM PQ (Figure 5B; Supplementary Figure S6).

Secondary mechanisms for OS management

The co-regulation of genes encoding rhodopsins, carotenoids, and gas vesicles with several known mechanisms to detoxify and manage ROS (e.g. in bc85—http://baliga.systemsbiology.net/drupal/content/egrin-oxidative-stress) suggested important functions for these processes in OS management (Figure 4). Culture characteristics of the perA and SOD mutants (Figure 6B and C) in conjunction with phenotypic analysis and transcriptomics further supported secondary protective functions for these functions and provided clues into their regulatory links to the primary protective mechanisms.

(a) Rhodopsins and carotenoids have distinct functions in peroxide and superoxide stress management: Pigments such as carotenoids, which are intermediates in synthesis of the retinal chromophore found in rhodopsins (Kushwaha et al, 1974), are known to act as antioxidants (Di Mascio et al, 1991). Consistent with this known function, the transcription of several genes involved in carotenoid biosynthesis [VNG1458G (crtB1), VNG1684G (crtI1), VNG1755G (crtI2), VNG1680G (crtB2), VNG1465G (bpr)] and one opsin VNG1467G (bop) were upregulated during and/or after exposure to H₂O₂ and PQ (Figure 2M and N). This is also consistent with earlier knowledge that transcriptional regulation of these genes is influenced by redox status of the cell (Betlach et al, 1989; Yang and DasSarma, 1990; Shand and Betlach, 1991; Baliga et al, 2001). Furthermore, visual inspection of stationary phase cultures revealed a distinctly bleached appearance of the SOD double mutant—an observation that was confirmed by the fourfold increase in the oxidative degradation rate of pigments during normal growth of this strain relative to the wild-type strain (Figure 6C). The specific mechanism by which carotenoids scavenge ROS is unknown, although it has been speculated that they act through interference with reactions of damaging oxidizing agents (Woodall et al, 1997; Davison et al, 2002). Regardless, an increased rate of pigment bleaching in the SOD double mutant, but not the ΔperA mutant suggests a specific protective function for carotenoids in defense against O₂ stress. This also partly explains the normal growth characteristics of the single and double SOD mutants under standard laboratory culturing conditions (Supplementary Figure S6). In contrast, depletion of all four rhodopsins [bacteriorhodopsin (bR), halorhodopsin (hR), sensory rhodopsins I and II (sRI and sRII)] as well as two transducers (HtrI and HtrII) in Pbo81, a strain constructed earlier for characterizing phototaxis mechanisms (Yao et al, 1994), resulted in increased peroxide toxicity (Figure 5C), but no difference in sensitivity to O₂⁻ (data not shown). In H. salinarum NRC-1, these proteins are involved in light-activated ion-pumping (bR and hR) and relocation of cells toward or away from particular wavelengths of radiation by phototaxis [sRI and II, and signal transducers (HtrI and HtrII)]. This is a new function for these integral membrane proteins. The specific mechanisms by which carotenoids and rhodopsins provide protection against H₂O₂ and PQ stress will require further investigation.

(b) Regulation of gas vesicle biogenesis is linked to peroxide stress: H₂O₂ and PQ treatment induced transcription of genes encoding structural components of gas vesicles. Further, EGRINOS suggested that under OS (especially H₂O₂ stress; see bc85) at least three of these genes [VNG6236G (gvpG2), VNG6237G (gvpF2), VNG6239G (gvpE2)] are co-induced with the dyp-type peroxidase (VNG0798H), a thioredoxin (VNG5076G (trxA1)), and a putative heat shock protein [VNG1801G (hsp1)]. The induction of purple membrane biogenesis and DMSO fermentation during OS, especially H₂O₂ treatment (Figure 2) suggests an OS-induced shift in physiology to one that is better suited for low oxygen conditions, so as to minimize the production of additional radicals. Induction of gas vesicle biogenesis under low O₂ tension is well known and speculated to be a mechanism used by H. salinarum NRC-1 for scavenging traces of O₂ through floatation (Yang and DasSarma, 1990; Schmid et al, 2007). However, in the context of OS, floatation because of vesicle
production would be problematic, as it would increase exposure to O$_2$. Intriguingly, deletion of perA abolished gas vesicle biogenesis resulting in a sinking phenotype (Figure 6B)—a phenomenon also displayed by cyanobacteria that are subjected to OS (Berman-Frank et al., 2004). In H. salinarum NRC-1, this appears to be mediated through post-transcriptional suppression of gas vesicle biogenesis despite increased transcript levels of these genes under conditions of increased H$_2$O$_2$ stress.

**Generalized and unique components of OSR**

It is generally accepted that a significant proportion of cellular responses to diverse types of environmental stress (metals, radiation, starvation, etc.) is shared and constitutes a generalized OS component. We investigated this idea quantitatively by evaluating the degree to which the EGRIN model constructed from responses to perturbations in diverse EFs (six metals, O$_2$, light, UV and γ-radiation) was able to predict transcriptional changes induced by H$_2$O$_2$ and PQ treatment. First, using the measured values of just 72 TFs, 9 EFs, and their regulatory influences in the EGRIN model, we predicted transcriptional changes in 80% of all genes in 5000 resampled datasets of 140 experiments each. These datasets were randomly assembled from a pool of 722 new experiments that were not used to construct the model and that did not include any OS experiments (Supplementary Figure S7). This analysis showed the remarkable robustness with which the EGRIN model consistently made accurate global predictions with a mean error (RMSD) of 0.36 ± 0.01. Next, we used the model to make predictions of global gene-expression changes in the 140 experiments (including controls) that specifically investigated transcriptional responses to treatments with H$_2$O$_2$ and PQ. Remarkably, the model predicted global gene-expression changes induced by OS with similar accuracy, suggesting that most regulatory phenomena triggered by OS are also active during responses to other EFs. Historically, such responses that are triggered by diverse environmental stresses are lumped into a loosely defined ‘general stress response.’ Although it might be true that a significant fraction of this general stress response is an indirect consequence of the highly interconnected nature of biological networks, many genes are most certainly triggered by a particular injury or dysfunction. Notwithstanding this observation, a closer investigation revealed OS-specific coordinate regulation of important genes within several regulons that were not observed in responses to any other EFs. Specifically, regulons within 22 of the 67 biclusters within EGRIN$_{OS}$ (residuals <0.45) are partially or completely disrupted ($R^2$ <0.35) in other environmental stress conditions such as treatments with sub-inhibitory doses of redox-active metals including Cu and Fe (Kaur et al., 2006), a sub-lethal dose of γ-radiation (Whitehead et al., 2006), or extreme changes in oxygen tension (Schmid et al., 2007) (Figure 7; Supplementary Table S10). The 288 genes within these 22 biclusters encode functions of detoxification, repair, Fe metabolism, nucleotide metabolism, and oligo/dipeptide ABC transporters. Although this analysis validates the notion that a significant fraction of cellular responses to diverse EFs does indeed constitute a generalized component, it also reveals specific regulatory mechanism(s) for the management of excessive OS. The regulon within bc84 is a case in point. Genes within this regulon encode frontline defense mechanisms that are coordinately upregulated by PQ treatment, and to a lesser degree by H$_2$O$_2$ treatment, but not by redox-active metals, γ-radiation, or extreme changes in oxygen tension. Co-regulation of Fe-trafficking genes within bc12 is another example of this OS-specific regulatory phenomenon (Figure 7). It will be important to understand natural circumstances that produce such extreme OS, so we can better characterize the functional relevance of such OS-specific regulatory circuits for survival in a hypersaline environment. Especially important will be the dissection of the hierarchy of regulatory networks that coordinate the generalized and specific components of OSR as a function of the degree of OS. The EGRIN$_{OS}$ model lends itself to such an analysis by providing conditional interrelationships among TF activities in the form of joint influences on both response components of the OSR.

**Summary and conclusion**

Given the ubiquitous nature of OS, it is not entirely surprising that most organisms have evolved multiple lines of defense—both passive and active. Although many of these mechanisms have been extensively characterized in other organisms, our integrated systems approach has uncovered additional protective mechanisms in H. salinarum (e.g. VNG0798H and VNG0018H). Further, the systems approach has also revealed a structure and hierarchy to the OSR through conditional regulatory associations among various components of the response (Figures 4 and 5). Using this integrated approach, we can begin to synthesize an understanding of the multi-tiered program for management of H$_2$O$_2$ and O$_2$ stress in H. salinarum NRC-1.

Briefly, H$_2$O$_2$ generated by normal metabolism is primarily handled by the peroxidase/catalase PerA; when H$_2$O$_2$ production exceeds detoxification capabilities of PerA, additional protection is provided by VNG0018H, rhodopsins, and VNG0798H, respectively (Figure 5). Uncontrolled accumulation of H$_2$O$_2$ such as on disruption of PerA causes cells to switch to anaerobic physiology, shutdown gas vesicle biogenesis, and sink away from oxygen (Figure 6). Coordinated upregulation of iron trafficking and management systems under severe H$_2$O$_2$ stress (bc12) might also serve to minimize Fenton reaction while keeping with the cellular demand for iron. O$_2$ stress, on the other hand, is primarily managed by SOD1 with additional marginal protection from SOD2. When challenged with a sub-lethal dose of O$_2$, H. salinarum NRC-1 increase the production of carotenoids to take advantage of their secondary function in scavenging ROS. Severe O$_2$ stress results in the co-regulation of several frontline defense and repair functions including sod1, msra, and dpsA (bc84) (Figure 3).

In addition to such O$_2$-specific responses, there was also evidence of crosstalk in responses to H$_2$O$_2$ and O$_2$ in the coordination of biclusters containing SOD1 (bc84) and VNG0798H (bc6) by the same set of regulatory influences (Figure 4). Likewise, two of the three peroxidases, VNG0018H and perA, also provide significant cross-protection against O$_2$.
stress (Figure 5). Although this is to be expected as ROS are readily inter-converted such as by dismutation of \( \text{O}_2 \) to \( \text{H}_2\text{O}_2 \), these network connections have also provided insights into the operational relationships in the crosstalk among two distinct OS protection mechanisms. These operational relationships are further extended by the model to other aspects of physiology. For instance, the model reveals that the simultaneous upregulation of genes within bc6 (\textit{VNG0798H}) and bc84...
(sod1, mcsra, dpsA) under certain OS conditions is coordinated with downregulation of cobalamin synthesis and oxidative phosphorylation (bc91) in a biologically meaningful manner (Figure 4). We have validated some aspects of the architecture of this OSR network by confirming physical protein–DNA interactions of six TFs with promoters of genes they were predicted to influence by EGRINOS (Figure 4B). Furthermore, we have also shown that deleting the TFs results in decreased transcript levels of predicted target genes and that disruption of this control can lead to decreased survival rate under OS (Figure 4C and D). It is notable that the five GTFs whose functions we have validated cannot directly sense ROS. This illustrates the importance of the EGRINOS architecture and the functions GTFs have therein to globally coordinate various processes during OSR. This systems level insight would not be possible without integration of diverse global datasets. Finally, by comparing across active regulatory programs under diverse environmental stresses, we have quantitatively determined processes that are specifically triggered by extreme OS (Figure 7).

Materials and methods
Organism and growth conditions
H. salinarum NRC-1 (wild type), H. salinarum NRC-1 ΔaurA3 (parent for knockout strains), and all gene knockout strains were grown in complex medium (CM: 250 g/l NaCl, 20 g/l MgSO4, 2 g/l KCl, 3 g/l sodium citrate, 10 g/l Oxoid brand bacteriological peptone) at 37 °C and 220 r.p.m. shaking in Innova9400 incubator (New Brunswick). CM was supplemented with 50 mg/l uracil for strains constructed from a ΔaurA3 background. Gene deletion mutants were constructed using a two-step in-frame gene replacement strategy as described earlier (Kaur et al., 2006) and rhodopsin-deficient strain, PhoA1, has been characterized earlier (Yao et al., 1994).

Growth and survival assay
All strains were grown in CM with continuous shaking (200 r.p.m.) at 37 °C until late log phase (OD₆₀₀ ≈ 0.8) and then further diluted into fresh medium to an optical density of 0.05. These cultures were then transferred to Honeycomb plate wells of Bioscreen C (Growth Curves USA, Piscataway, NJ). Different amounts of PQ (0, 0.125, 0.25, and 0.5 mM—final concentrations) or H₂O₂ (0, 4, 5, 6, and 7 mM—final concentrations) were added into individual wells. Samples were incubated at 37 °C with continuous shaking and growth was monitored for 48 h in Bioscreen. For survival assay, H. salinarum NRC-1 was exposed to either 0–100 mM H₂O₂ or 0–8 mM PQ, whereas knockout strains were exposed to only 25 mM H₂O₂ or 4 mM PQ for 2 h treatment. Cell viability was determined by counting colonies on agar plates. Sensitivity of ΔaurA3, ΔperA, AVNC0798H, and PhoA1 were also tested with different concentrations of H₂O₂ (5, 15, 25 mM). Three independent measurements were made for each mutant.

Exposure to H₂O₂ and PQ for RNA preparation and microarray analysis
Two time courses were run to determine the transcriptional responses to (1) constant stress and (2) recovery of H. salinarum NRC-1 to H₂O₂ and PQ. Mid-log phase cultures grown in flasks were treated with sub-lethal concentrations of 25 mM H₂O₂ or 4 mM PQ and incubated at 37 °C with shaking for up to 240 min. During constant stress, culture aliquots (~4 ml) were collected over a time course (~1, 5, 10, 20, 40, 80, and 160 min), centrifuged (16 000 × g, 90 s), and flash frozen. For recovery, cells were first treated for 2 h with either 25 mM H₂O₂ or 4 mM PQ, recovered by centrifugation, washed, and re-suspended in CM. Cultures were returned to the incubator with shaking and samples were taken at 0, 10, 20, 30, 40, 50, 60, 120, and 240 min and processed as described earlier. Analysis of temporal transcriptional changes (~1, 5, 10, 20, 40, 80, 160, and 320 min) to sub-inhibitory concentrations of PQ (0.25 mM) was performed using the BioFlo110 modular bench-top fermentor (New Brunswick Scientific). Total RNA was prepared using the Absolutely RNA kit (Stratagene) according to manufacturer’s instructions. Microarray slide fabrication, labeling with Alexa547 and Alexa647 dyes (Molecular Probes and Kreatech BV), hybridization, and washing were performed as described earlier (Baliga et al., 2004). Raw intensity signals were processed and resultant data were median normalized and evaluated for statistical significance of differential expression with significance of microarray (SAM) and variability and error estimates (VERA) algorithms (Ideler et al., 2000). In constant datasets, mRNA log₁₀ ratios were normalized to first condition (control) and data was filtered based on fold change >0.05, 0 = 15 in at least one condition. In recovery sets, data was filtered (fold change > 0.05, λ = 15) and compared with temporal changes in control experiments. A total of ~1400 genes met these criteria. Genes were further classified into different functional groups according to Kyoto Encyclopedia of Genes and Genomes, PFAM (http://pfam.sanger.ac.uk/), and clusters of orthologous groups (http://www.ncbi.nih.gov/COG). Analysis of microarray results was performed using hierarchical clustering and Dynamic Regulatory Events Miner (Ernst et al., 2007), which uses a hidden Markov model to construct dynamic models for condition-specific gene regulation. The complete microarray dataset is available at http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE17515.

H₂O₂ production during aerobic growth
H₂O₂ production was measured with Amplex Red H₂O₂ assay (Molecular Probes—A22188). Late log phase cultures of ΔaurA3, ΔperA strains were diluted 1:100 in fresh CM and grown for six days. H₂O₂ levels were measured along the growth curve at different time points (21, 29, 36, 48, 72, and 144 h). The rate of H₂O₂ was normalized to the number of cells. The relation between OD₆₀₀ and cell number was calculated by counting cells in a flow cytometer.

Pigment analysis
Strains (ΔaurA3, Δsod1, ΔsodL, Δsod1/Δsod2) were grown in CM media at 37 °C until late stationary phase. Aliquots (1 ml) were withdrawn from cultures at different time points (~24 h intervals), centrifuged at 16 000 × g for 2 min. Pigments were extracted from cell pellets with 90% cold acetone and analyzed with UV-VIS spectrophotometer (Beckman).

Figure 7 Coordination of frontline defense mechanisms under extreme OS. (A) Degree of co-regulation of genes within 67 biclusters (residuals < 0.45) in diverse environmental stress conditions was evaluated by calculating correlations (ρ) among their transcript level changes in those experiments. The ρ-values were hierarchically clustered to identify the generalized and specific components of OSR. (B) Pearson’s correlation (ρ) among mean and variance-normalized transcriptional changes for four representative biclusters are shown. Biclusters 31 and 37 are examples of the generalized stress response component of OSR with co-regulation across most environmental conditions. In contrast, co-regulation of genes within biclusters 12 and 84 is much more significant under severe OS conditions. For instance, genes of bc84 are co-regulated under severe OS resulting from treatment with sub-lethal dose PQ and to a lesser degree H₂O₂, but not on irradiation with sub-lethal dose of γ-irradiation (Whitehead et al., 2006), subjecting cells to sudden and extreme changes in O₂ tension (Schmid et al., 2007) or treatment with sub-inhibitory dose of transition metals (Kaur et al., 2006). Genes within bc12, on the other hand, were better co-regulated on treatment with H₂O₂. Genes within both biclusters (especially bc84) encode important functions associated with frontline defense mechanisms that provide protection against OS (see Figure 3 and http://baliga.systemsbiology.net/drupal/content/eegrin-oxidative-stress for characteristics of biclusters). (C) A specific example of OS-specific co-regulation: two genes of bc84, sod1 and msra, are co-induced under severe OS—especially with PQ treatment—but not coordinately controlled by other stressful environmental conditions.
Gas vesicles visualization

Aliquots were harvested at various time points in Δura3 and ΔperA strains and further diluted to an OD600 by the addition of 0.25% formaldehyde (final concentration) and imaged with phase contrast microscopy.

Calculation of significance of enrichment

List of oxygen responsive genes was generated with principal component analysis of oxygen microarray experiment from earlier study (Schmid et al., 2007), which resulted in two categories of genes: one correlated (oxic genes—105) and other anti-correlated (anoxic genes—110) with oxygen. P-values were computed for enrichment of both oxic and anoxic genes among all OS responsive genes based on hyper-geometric distribution.

Motif search

Motifs conserved for various functions such as redox (CX2S), Fe–S (CX3CX2CX3C and CX3CX2C), conserved cysteines residues of oxyR protein (CX2C), and metal containing cysteine residues (CX2C) along with PAS (PF00989, PF00785), GAF (PF01590), and MCF (PF00015)—signaling domain were identified from literature (Zheng and Storz, 2000; Fomenko and Gladyshev, 2002; Paget and Buttner, 2003; Fontecave, 2006). MatLab scripts were written to search the proteome for matches to one or more of these conserved motifs (Supplementary Figure S4; Supplementary Table S5).

Accession sites

Access to modeling results online @: http://baliga.systemsbiology.net/drupal/content/eegrin-oxidative-stress.

Supplementary information

Supplementary information is available at the Molecular Systems Biology website (http://www.nature.com/msb).

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Conflict of interest

The authors declare that they have no conflict of interest.

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Gas vesicles visualization

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