Defining the binding determinants of *Shewanella oneidensis* OxyR: implications for the link between the contracted OxyR regulon and adaptation

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Running title: OxyR regulation in *S. oneidensis*

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**ABSTRACT**

It is well established that OxyR functions as a transcriptional activator of the peroxide stress response in bacteria, primarily based on studies on *Escherichia coli*. Recent investigations have revealed that OxyRs of some other bacteria can regulate gene expression through both repression and activation or repression only; however, the underlying mechanisms remain largely unknown. Here, we demonstrated in γ-proteobacterium *Shewanella oneidensis* that regulation of OxyR on expression of major catalase gene *katB* in a dual-control manner through interaction with a single site in the promoter region. Under non-stress conditions, *katB* expression was repressed by reduced OxyR (OxyR\(_{\text{red}}\)), whereas when oxidized, OxyR (OxyR\(_{\text{oxi}}\)) outcompeted OxyR\(_{\text{red}}\) for the site because of substantially enhanced affinity, resulting in a graded response to oxidative stress, from repression, derepression, to activation. The OxyR-binding motif is characterized as a combination of the *E. coli* motif (tetranucleotides spaced by heptanucleotide) and palindromic structure. We provided evidence to suggest that the *S. oneidensis* OxyR regulon is significantly contracted compared to those reported, probably containing only five members that are exclusively involved in oxygen reactive species scavenging and iron sequestering. These characteristics likely reflect the adapting strategy of the bacteria that *S. oneidensis* represents to thrive in redox-stratified microaerobic and anaerobic environments.

The ability of bacteria to alter gene expression patterns in response to environmental stresses is critical for their survival. This is particularly true about oxidative stress because its causing agents, reactive oxygen species (ROS), are omnipresent: in addition to those coming from environments, they are generated endogenously as metabolic by-products of cellular oxygen respiration (1). ROS, including superoxide (O\(_2^–\)), hydrogen peroxide (H\(_2\)O\(_2\)), and hydroxyl radical (•OH), damage biomolecules such as lipids, proteins and DNA (1). When ROS levels exceed safe limits, sensing and responding systems are triggered to coordinately regulate expression of a set of genes to ensure that the ROS concentrations are restrained at an acceptable level and...
damages are promptly repaired (2). The primary members of these genes encode ROS detoxification enzymes (alkylhydroperoxide reductase (Ahp, renamed NADH peroxidase), catalases, and various peroxidases), iron-sequestering proteins (Dps in particular), and oxidative damage repairing macromolecules (3).

One of the major ROS sensing and responding systems is OxyR, a LysR-type transcriptional regulator (LTTR) consisting of an N-terminal DNA-binding domain (DBD) and C-terminal regulatory domain (RD) (2,4). In model bacterium *Escherichia coli*, OxyR in a tetrameric arrangement is able to activate the transcription of its target genes through the DBD extremely rapidly, within 1-2 min after *E. coli* cells are exposed to H$_2$O$_2$ (5). The activation of OxyR by H$_2$O$_2$ oxidation is through the formation of an intracellular disulfide-bond between the two conserved cysteine residues located in the RD. This induces structural changes of the RD, leading to conformational rearrangement of the DBD to alter the DNA-binding affinity (6,7). OxyR-binding motif in *E. coli* is characterized by four OxyR-binding tetranucleotide sequences spaced by heptanucleotides (consensus, ATAGntnnnanCTAT-N$_7$-ATAGntnnnanCTAT) (8). It has been proposed that each subunit of the OxyR homotetramer specifically binds to one of four tetranucleotide sequences and because of the spacing, the four subunits interact with four adjacent major grooves on one side of the DNA helix (6,8).

In recent years, studies into OxyRs of other bacteria have revealed some variations to the *E. coli* OxyR model. In *Deinococcus radiodurans*, OxyR is activated by oxidation at the conserved single cysteine residue to a sulfenic acid under peroxide stress (9). In several bacterial species such as Neisseria, OxyRs function in a dual-control manner (both a repressor and an activator) for major H$_2$O$_2$ scavenging proteins and an activator only for other members of their regulons; as a consequence, OxyR-deficient strains are more resistant to H$_2$O$_2$ (10-13). More surprisingly, *Corynebacteria* OxyRs function primarily as a repressor for more than 20 genes, including those for ROS detoxification enzymes and iron-sequestering proteins (14-16). In general, the majority of OxyRs are homologous in sequence (~30-35% in identity) and similar in structure, and thus functionally exchangeable (10-16).

*Shewanella*, a group of Gram-negative facultative γ-proteobacteria, thrive in redox-stratified environments and are renowned for their respiratory versatility (17). Because of the potential application in bioremediation, biogeochemical circulation of minerals and bioelectricity, these bacteria have been intensively studied, especially the model species *S. oneidensis* (18). The *S. oneidensis* OxyR (SoOxyR) resembles those of *Neisseria* in that it represses expression of catalase gene katB under non-stress conditions but functions as an activator for all of its target genes when cells are challenged by H$_2$O$_2$ (19). Nevertheless, three unexpected phenotypes resulting from the SooxyR disruption were observed (11). First, although the SooxyR mutant under non-stress conditions degrades H$_2$O$_2$ more rapidly, it still carries defects in both viability (plating defect) due to increased sensitivity to H$_2$O$_2$ and growth (18, 19, 20), phenotypes reported in bacteria with OxyR being a positive regulator, such as *E. coli* (21; 22). Second, SoOxyR is also able to respond to organic peroxides (OP), functionally intertwined with OhrR, a regulator specific for OP (23). Third, SoOxyR is unable to complement the *E. coli* oxyR mutation and *vice versa* although they share a comparable sequence similarity as mentioned above (11, 13, 19). Hence, SoOxyR apparently mediates...
cellular response to oxidative stress with unprecedented mechanisms.

In our continued efforts to determine factors accountable for the characteristics of the SoOxyR mutant, we took on in this study to assess the biochemical properties of SoOxyR. We demonstrate that SoOxyR proteins, present in reduced (SoOxyR<sub>oxi</sub>) and oxidized (SoOxyR<sub>red</sub>) forms, recognize a single site featured by a combination of the OxyR-binding motif of <i>E. coli</i> and palindromic structure. When present, SoOxyR<sub>oxi</sub> dictates regulation because of higher affinity for the site than SoOxyR<sub>red</sub>. Consequently, SoOxyR<sub>oxi</sub> in excess exerts great inhibitory effects on growth, but interestingly not on viability. Furthermore, evidence is provided to suggest that the SoOxyR regulon appears contracted significantly, having only five members.

RESULTS

Recombinant His-tagged OxyR proteins exist in reduced and oxidized forms - <i>S. oneidensis</i> OxyR, as a H<sub>2</sub>O<sub>2</sub>-responsive transcriptional regulator, empowers cells to confront oxidative stress by regulating gene expression through reversible formation of intermolecular disulfide bond (19). In order to characterize the biochemical properties of this regulator in more detail, we expressed recombinant SoOxyR with hexahistidine(his<sub>6</sub>)-tag at the N-terminal in <i>E. coli</i>. A large fraction of the protein was soluble, from which the protein was purified by Ni<sup>2+</sup>-affinity chromatography, as revealed as two bands of appropriate molecular mass on SDS-PAGE (Fig. 1A). The scenario suggests that the protein may exist in both reduced and oxidized forms, SoOxyR<sub>red</sub> and SoOxyR<sub>oxi</sub> respectively. To confirm that both bands are SoOxyR proteins, they were excised from an SDS-PAGE gel, digested with trypsin, and the resulting peptides were analyzed with liquid chromatography mass spectrometry (LC-MS/MS). The result verified their identities in that the peptides matched SoOxyR with 78% coverage of the expected peptides (Fig. S1). To investigate that oxidation underlies the two bands, the protein samples were treated with reducing agent dithiothreitol (DTT) of various concentrations and then applied to SDS-PAGE (Fig. 1B). Clearly, SoOxyR<sub>oxi</sub> can be converted to SoOxyR<sub>red</sub> by DTT and complete conversion was achieved with 3.0 mM. We then performed chemical crosslinking assays to evaluate whether SoOxyR forms oligomers at high concentrations (1). Indeed, when treated with crosslinker ethylene glycol bis-succinimidylsuccinate (EGS), oligomerization of SoOxyR, dimerization in particular, was evident (Fig. 1C). These results indicate that SoOxyR, likely functioning in an oligomeric structure, exist in both reduced and oxidized forms.

SoOxyR<sub>oxi</sub> always functions as an activator whereas SoOxyR<sub>red</sub> is conditional as a repressor - <i>E. coli</i> OxyR senses H<sub>2</sub>O<sub>2</sub> via the reversible formation of disulfide bond between the two conserved cysteines (Cys-199 and Cys-208). In SoOxyR, the counterparts of these two cysteines are Cys-203 and Cys-212 respectively; mutation of either residue to serine locks SoOxyR into the reduced form (19, 24). In addition, SoOxyR<sup>L197P</sup> mutant is found to be locked into the oxidized state (25). To determine physiological activity of SoOxyR of different states, we assessed ability of SoOxyR variants, SoOxyR<sup>WT</sup> (wild-type), SoOxyR<sup>C203S</sup>, and SoOxyR<sup>L197P</sup>, to complement the SooxyR null mutant (ΔSooxyR). We placed the SooxyR alleles under the control of isopropyl-β-D-thiogalactopyranoside (IPTG)-inducible promoter P<sub>_tac</sub> within pHGE-P<sub>_tac</sub> and the resulting vectors were introduced into ΔSooxyR. Spotting assays of series dilution cultures were performed to examine the
plating defect resulting from the SoOxyR loss on LB plates as it is the most dramatic phenotype (18, 19). Because the promoter is slightly leaky (26, 27), both SoOxyR<sup>WT</sup> and SoOxyR<sup>L197P</sup> produced in the absence of IPTG were sufficient to fully correct the plating defect of ΔSoOxyR, but SoOxyR<sup>C203S</sup> could not. Additionally, induced SoOxyR<sup>C203S</sup> reduced the viability of ΔSoOxyR cells as evidenced by decreased survival of undiluted cultures on LB plates (Fig. 2A). We also noticed that SoOxyR<sup>L197P</sup> in excess impeded growth when IPTG was at 0.02 mM, with much more severe effects being observed from 0.2 mM. Overproduced SoOxyR<sup>WT</sup> appeared to inhibit growth too, but the effect was much less significant.

In parallel, impacts of the SoOxyR variants on H<sub>2</sub>O<sub>2</sub> sensitivity was assessed by diffusion disc assay, which was carried out in the presence of 0.02 mM IPTG. The results revealed that the ΔSoOxyR strain had an inhibition zone smaller (11 mm) than the wild-type (16 mm) (Fig. 2B, S2), a phenomenon reported before (19). While production of SoOxyR<sup>C203S</sup> substantially augmented the zone of ΔSoOxyR, approximately 34 mm in diameter, SoOxyR<sup>L197P</sup> virtually eliminated the zone. Given that in <i>S. oneidensis</i> KatB is the catalase predominantly accounting for decomposing H<sub>2</sub>O<sub>2</sub> and therefore resistance (19) (Fig. 2B), we subsequently evaluated influence of SoOxyR variants on KatB production with catalase staining (Fig. 2C). Production of KatB in the wild-type was responsive to H<sub>2</sub>O<sub>2</sub>, being substantially increased when treated with H<sub>2</sub>O<sub>2</sub>. Without SoOxyR, <i>S. oneidensis</i> cells lost ability to respond to H<sub>2</sub>O<sub>2</sub>, stably producing KatB at intermediate levels, between those of the wild-type under non-induced and induced conditions because of derepression (explaining why ΔSoOxyR has more robust H<sub>2</sub>O<sub>2</sub>-scavenging capacity as shown in Fig. 2B). These data are in excellent agreement with previous findings (18, 19). In ΔSoOxyR/SoOxyR<sup>L197P</sup>, the amount of KatB produced was drastically increased, migrating as the supercomplex on the gel (Fig. 2C). In contrast, KatB in ΔSoOxyR/SoOxyR<sup>C203S</sup> was undetectable either in the treated or untreated cells, indicating that KatB expression is repressed by SoOxyR<sup>C203S</sup> consistently. For further confirmation, catalase activities of these samples were assayed. The cell extracts the same as for catalase staining were mixed with 10 mM H<sub>2</sub>O<sub>2</sub>. Decomposition of H<sub>2</sub>O<sub>2</sub> was measured at 15-s time intervals for a total time of 3.5 min to calculate catalase activity. As shown in Fig. 2C, the results correlated well with those of catalase staining.

We further examined impacts of these SoOxyR variants on expression of <i>ahpC</i> that is subject to SoOxyR activation only by qRT-PCR (Fig. 2D). Like katB, ahpC was responsive to H<sub>2</sub>O<sub>2</sub> in the wild-type, with substantially enhanced transcription upon H<sub>2</sub>O<sub>2</sub>-treatment. In ΔSoOxyR, although the responsiveness was abolished, ahpC differed from katB in that its transcription was down-regulated, indicating that SoOxyR does not function as a repressor for <i>ahpC</i>. This was supported by that SoOxyR<sup>C203S</sup> failed to elicit detectable impacts on <i>ahpC</i> transcription. Expectedly, we observed that transcription of <i>ahpC</i> in SoΔOxyR/SoOxyR<sup>L197P</sup> was substantially up-regulated. All together, these results indicate that SoOxyR proteins are present in the reduced and oxidized states, both of which mediate regulation: SoOxyR<sub>oxi</sub> appears to activate the regulon in general but SoOxyR<sub>red</sub> only works for some as a repressor.

**Analysis of the promoter region of the katB gene** - Data presented thus far have demonstrated that katB expression is constitutively activated by SoOxyR<sup>L197P</sup> and
repressed by SoOxyR<sup>C203S</sup>. We wonder whether SoOxyR interacts with different DNA sites for these two opposing effects on katB expression. To this end, we first mapped the transcription start site (TSS) for the katB gene with 5′-RACE (Fig. 3A). The result revealed one major elongated primer product initiating at the position corresponding to A-44, the adenine 44 bp upstream of the translation initiation codon ATG (Fig. 3B).

We then used an integrative lacZ-reporter system to evaluate activity of a series of katB promoters. DNA fragments of various lengths upstream of the katB coding sequence (from -362, -232, -188, -152, -108, or -70 to -1) were amplified and cloned into the reporter vector pHEGI01 (Fig. 3C). The resulting vectors, verified by sequencing, were introduced into relevant <i>S. oneidensis</i> strains for integration and subsequent removal of the antibiotic marker (28). β-galactosidase assay revealed that activities of the fragments no less than 188-bp were comparable under any given condition, indicating that they are the same in functionality; to simplify description, only data from 188-bp (P<sub>katB-188</sub>) were shown (Fig. 3C). P<sub>katB-188</sub> was responsive to H<sub>2</sub>O<sub>2</sub> in the wild-type but not in ΔSooxyR. Moreover, P<sub>katB-188</sub> activities in the untreated and treated wild-type cells were substantially lower and higher than those in ΔSooxyR respectively. These data are consistent with the catalase staining results (Fig. 2C), indicating that this 188-bp fragment contains all elements required for both repression and activation of SoOxyR. In contrast, P<sub>katB-152</sub> and P<sub>katB-108</sub> exhibited considerable activities but were unable to respond to H<sub>2</sub>O<sub>2</sub>, implicating that these two promoter fragments are functioning and lack the elements for SoOxyR regulation. Activity of P<sub>katB-70</sub> was barely detectable, indicating that the promoter in the fragment is destroyed. These results suggest that the SoOxyR binding sites lie between -108 and -188 bp upstream of the katB gene.

**Mapping the binding sites of OxyR<sub>oxi</sub> and OxyR<sub>rede</sub>** EMSA was performed to detect the binding ability of purified his<sub>6</sub>-tagged SoOxyR<sup>WT</sup>, SoOxyR<sup>C203S</sup> and SoOxyR<sup>L197P</sup> to a katB promoter fragment covering -300 to -1 in which the predicted OxyR-binding sites are centered. All SoOxyR variants bound well to this fragment in comparison with negative control P<sub>16s</sub> (promoter sequence of 16s rRNA gene) (Fig. 4A), indicating that the sites with which SoOxyR variants interact are concluded. It is noteworthy that SoOxyR variants OxyR<sup>L197P</sup> had the strongest affinity for the katB promoter, giving apparently band shift even at 2.5 nM, contrasting SoOxyR<sup>C203S</sup>, with which band shift was observed at 50 nM and above.

In order to pinpoint the precise binding sequences of SoOxyR<sup>WT</sup>, SoOxyR<sup>C203S</sup> and SoOxyR<sup>L197P</sup>, DNA footprinting experiment with the DNA fragment was performed. The fragments were labeled with 6-Carboxyfluorescein (6-FAM), incubated with increasing concentrations of SoOxyR variants, and subjected to DNase I digestion. Upon addition of 4.5 μg SoOxyR<sup>WT</sup>, a region of protection corresponding to the area from -126 and -173 with respect to the ATG became clearly evident (Fig. 4B). Interestingly, SoOxyR<sup>L197P</sup> and SoOxyR<sup>C203S</sup> protected almost the same region as SoOxyR<sup>WT</sup>, but the region expanding forward 7~10 bp with respect to the direction to the ATG (Fig. 4B). It was evident that the protected region contains a perfect palindrome sequence, TTAAAGTAGATT(GTAAG)AATCTACTTTAA. In contrast, whether the region also covers a sequence pattern mimicking the OxyR-binding motif of <i>E. coli</i> (ATAGntnnnanCTAT-N<sub>7</sub>-ATAGntnnnanCTAT) was unknown as this...
motif cannot be easily identified by sequence analysis.

OxyR<sub>oxi</sub> outcompetes OxyR<sub>red</sub> in DNA-binding - Since EMSA assays do not measure binding under equilibrium conditions, fluorescence anisotropy was chosen to examine DNA binding in a quantitative manner at equilibrium (29). Fluorescein-labeled, 60-bp oligonucleotides that contain the entire binding-motif were synthesized. As shown in Fig. 5A, the fluorescence anisotropy of the target DNA increased with increasing amounts of SoOxyR<sup>L197P</sup> and SoOxyR<sup>C203S</sup>, indicating that both proteins bound to this DNA probe. The ability of these proteins to bind the labeled DNA was clearly different based on the <i>K<sub>d</sub></i> values calculated from the anisotropy data. SoOxyR<sup>L197P</sup> bound to the probe with a <i>K<sub>d</sub></i> of 0.261 ± 0.03 µM whereas SoOxyR<sup>C203S</sup> bound with a <i>K<sub>d</sub></i> of 1.041 ± 0.12 µM. The significant (<i>P</i> < 0.001; <i>t</i> test) difference in <i>K<sub>d</sub></i> values indicates that SoOxyR<sub>oxi</sub> binds to its target DNA sequence with an affinity of about 4 times higher than that of SoOxyR<sub>red</sub>.

Given these differences, we reasoned that high-affinity SoOxyR<sub>oxi</sub> would overshadow SoOxyR<sub>red</sub> in physiological impacts when they coexist in the cell. To test this, we calibrated promoter activity of SooxyR with the integrative lacZ reporter in comparison with integrative vector carrying P<sub>lac</sub>-lacZ (Fig. S3). When P<sub>lac</sub> is on the chromosome, its activity in the presence of 0.02 mM IPTG was comparable to that of P<sub>oxyR</sub> under normal conditions. Constructs of SoOxyR<sup>L197P</sup> and SoOxyR<sup>C203S</sup> whose expression was driven by P<sub>lac</sub> were then introduced into the wild-type and integrated onto the chromosome. In the presence of IPTG at varying concentrations, impacts of SoOxyR<sup>L197P</sup> and SoOxyR<sup>C203S</sup> on growth of the wild-type were assessed. As shown in Fig. 5B, SoOxyR<sup>C203S</sup> expressed from the IPTG-inducible promoter had undetectable effects on growth, implicating that it could not interfere with SoOxyR<sup>WT</sup> in regulation. When produced at low levels (IPTG at 0.01 mM), effects of SoOxyR<sup>L197P</sup> were not evident. However, in the presence of IPTG at 0.02 (equivalent to the oxyR native promoter) and 0.2 mM, SoOxyR<sup>L197P</sup> inhibited growth, and inhibitory effects increased with levels of the inducer. The impact is on growth rather than viability because the growth defect resulting from SoOxyR<sup>L197P</sup> production remained in the presence of catalase. These data support that SoOxyR<sub>oxi</sub> outcompetes SoOxyR<sub>red</sub> in regulation in vivo.

<i>S. oneidensis</i> OxyR-binding motif is complex - Although LTTRs bind to 15-17 bp palindromic regions as dimeric proteins proposed initially, they are active in tetramer form and protect large regions of DNA (up to 60 bp) (4). In line with this, OxyR binding motifs determined to date are up to 50 bp and low in conservation (10, 13, 14, 30, 31). In order to determine the pattern of the binding motif of SoOxyR, we tested promoter regions of H<sub>2</sub>O<sub>2</sub>-responsive genes, including ahpC, ccpA, dps, and katG-1, for <i>in vitro</i> binding of purified SoOxyR<sup>L197P</sup> protein by EMSA. All of the target DNA probes produced distinct retarded bands when SoOxyR proteins were added (Fig. 6A), validating that SoOxyR directly binds to these promoters. From the promoter regions of katB, katG-1, ahpC, dps, and ccpA, we deduced the SoOxyR consensus sequence with AlignACE and MDScan. Three putative binding motifs were obtained and they are all palindrome-like sequences and nested (Fig. 6B). Importantly, these motifs reside in the region defined by DNase I footprinting presented above (Fig. 4B). Motif SoOxyR-M13 (13-bp, GATTGTAAGAATC in katB promoter region) is in the central, which extends 8 and 13 bp on both ends to form SoOxyR-M29 and SoOxyR-
M39 respectively. Although the bioinformatics tools failed to identify a motif resembling that of EcOxyR-binding motif, putative tetranucleotide sequences were manually marked out from these sequences (Fig. 6B).

To test the pattern of the SoOxyR-binding motif, we constructed a series of promoter mutants, \( P_{katB} \)-M1-M6, to drive expression of \( E. coli \ lacZ \) (Fig. 6C). M1 and M2 were designed to test the importance of the 13-bp central palindromic sequence (SoOxyR-M13), lacking (by deletion) and losing (by point mutations) the feature respectively. β-galactosidase assays revealed that M1 lost response to \( H_2O_2 \) both negatively and positively, resembling that of \( P_{katB} \) in \( ΔSoOxyR \), whereas M2 retained such ability, albeit significantly compromised (Fig. 6D). M3 aimed to estimate the essentiality of the 7-bp linker between the first two tetranucleotide sequences while M4 altered structure of the 29-bp palindrome sequence (SoOxyR-M29).

The change in the length of the linker (M3) abolished the responding ability of the promoter to \( H_2O_2 \), but M4 largely behaved as the wild-type (Fig. 6D). M5 and M6 were used to test the significance of the first and last tetranucleotide sequences, which have highest and lowest conservation respectively. The promoter activities showed that destruction of the first tetranucleotide sequence (M5) largely rendered SoOxyR inability to activate \( katB \) expression upon \( H_2O_2 \) treatment whereas M6 only slightly affected the characteristics of the promoter (Fig. 6D). These data, collectively, suggest that the SoOxyR-binding motif is likely composed of four tetranucleotide sequences separated by 7-bp linker, the last of which appears less conserved with respective to regulation.

Additionally, the palindromic sequence of the central 13-bp but not the extended 29-bp is critical for regulation. We then constructed the SoOxyR binding weight matrix based on the experimentally verified SoOxyR binding sequences (Fig. 6B). Screening for the other SoOxyR binding sites in \( S. oneidensis \) genome was carried out using RSAT (32). In total, 11 putative SoOxyR binding sites were identified with weight values greater than 7, a cut-off for reliable prediction (Table 1). Except for genes like \( ahpC, ccpA, dps, katG-1, \) and \( katB \) that have been verified by EMSA, all other genes on this list except for \( def \) (polypeptide deformylase) were not reported to be associated with oxidative stress response (1). More importantly, weight values of putative motifs for these genes are relatively low (highest, 14.4); there is a sharp gap between them and those of verified sites such as \( katB \) (lowest, 26.6). Although further investigation is needed, these data implicate that \( S. oneidensis \) OxyR possesses an extremely contracted regulon compared with that of \( E. coli \), which consists of more than 30 genes (30).

**DISCUSSION**

In many Gram-negative bacteria, oxidative stress response is critically mediated by \( H_2O_2 \)-responsive transcriptional regulator OxyR, a member of the LTTR family (2). Upon induction by \( H_2O_2 \), OxyR undergoes a conformational change, allowing the oxidized regulator to function as an activator for genes encoding proteins involved in protection against ROS, a scenario best illustrated in \( E. coli \) on which most of our understanding about this regulator is built (1, 33). However, this turns out to be one side of the regulatory effects about OxyR proteins. In recent years, OxyRs that work as a repressor only or through a dual-control mechanism have been found, in *Corynebacteria* and *Neisseria* as exemplary bacteria respectively (9-16). Clearly, the \( S. oneidensis \) OxyR resembles
those of *Neisseria*, working as both a repressor and an activator. Nevertheless, it carries novel features that have never been observed from OxyR proteins studied to date, including compromised resistance to exogenous H$_2$O$_2$, intertwined regulation with OhrR, and non-exchangeable functionality with *E. coli* OxyR (18-20, 23).

With SoOxyR variants that are locked in reduced and oxidized forms, we verified that SoOxyR$_{\text{red}}$ exclusively functions as an activator whereas SoOxyR$_{\text{oxi}}$ as a repressor is conditional, repressing *katB* expression under non-stress conditions only. In the wild-type cultivated without H$_2$O$_2$, *katB* is expressed at the basal level, which is so low that KatB catalase activity is barely detectable (Fig. 2C). Although deficient in activation, SoOxyR$_{\text{C203S}}$ (SoOxyR$_{\text{red}}$) was fully functional for H$_2$O$_2$-independent repression, a feature consistent with the proposal that SoOxyR acts as a repressor when not in its activated form (19). Depletion of SoOxyR prompts *katB* expression to a constitutive intermediate level, which can be substantially elevated further when oxidized SoOxyR is present. Clearly, this occurs through direct binding of SoOxyR to the promoter region of *katB* based on the mutational analysis of the *katB* promoter variants (Fig. 6).

Although SoOxyR binding motifs determined to date are low in sequence conservation, a feature in common is that they are relatively long (up to 60 bp) (4,10,13, 14,30,31). For repressor-only *Corynebacterium* OxyRs, OxyR-binding regions identified by EMSA are ~50-bp long with multiple T-N$_7$-A motifs of very low sequence similarities, a characteristic for sequences recognized by LTTRs (15). In the case of activator only and dual-control OxyRs, OxyR-bind motifs are primarily characterized by four tetranucleotide sequences (ATAG) spaced by heptanucleotides (8, 13). It is worth noting that OxyR-binding motifs of some bacteria, such as *Pseudomonas aeruginosa*, have been proposed to be 15-bp (31). However, these motifs, at least in the case of *P. aeruginosa*, reside in the middle of 37-bp DNA fragments resembling the *E. coli* OxyR-binding consensus sequence (8, 31).

Data presented here indicate that *S. oneidensis* OxyR-binding motifs corroborate the model of four tetranucleotide sequences (ATAG) spaced by heptanucleotides (Fig. 6). Disruption of tetranucleotide sequences except for the last one and the distance in between abolishes both repression and activation. The last tetranucleotide sequence in the *katB* promoter region is less critical, which may be due to low conservation among target promoters. Intriguingly, there are novel features in *S. oneidensis* OxyR-binding motifs. Two nested palindromic sequences, 13-bp and 29-bp, are located in the middle of the sites protected by SoOxyR from DNase I digestion. While the palindrome feature of the 13-bp sequence is important for regulation, the 29-bp one only exhibits a slight impact. Seemingly, these palindromic features are dispensable for repression of SoOxyR$_{\text{red}}$. Nevertheless, the 13-bp palindromic sequence may underlie, at least in part, functional discrepancy between OxyRs of *E. coli* and *S. oneidensis*. However, this merits further investigation.

All OxyR proteins examined to date, regardless of their regulatory effects, are capable of binding to promoter regions of their target genes in both reduced and oxidized forms (8, 13, 15). Given that H$_2$O$_2$ can be generated as a metabolic by-product of cellular oxygen respiration and abiotically (18), cells are living in an environment where a balance between the production and removal of ROS is maintained. Therefore, it is conceivable that OxyR proteins in reduced and oxidized forms co-exist in the cell (Fig. 1).
An obvious strategy for OxyR regulation is that OxyR<sub>red</sub> and OxyR<sub>oxi</sub> interact with the same site but differ from each other in binding-affinity. In <i>E. coli</i>, OxyR<sub>oxi</sub> has greater affinity than OxyR<sub>red</sub> to the consensus sequence (8, 33). In this study, we showed that the strategy is also employed by dual-control OxyR. In <i>vitro</i> binding assay revealed that OxyR<sub>oxi</sub> exceeds OxyR<sub>red</sub> in binding affinity more than 4 times (Fig. 5A). In parallel, <i>in vivo</i> analysis demonstrated that OxyR<sub>oxi</sub> overwhelms OxyR<sub>red</sub> in their regulatory effects when both proteins are produced at similar levels (Fig. 5B). It is worth mentioning that OxyR in excess negatively influences growth but not viability. We have previously shown that growth defect of the <i>oxyR</i> mutant is largely attributable to reduced efficacy of oxygen respiration, indirect effects of the <i>oxyR</i> mutation (20). We speculate the same mechanism accountable for the growth inhibition of excessive OxyR; efforts to test this notion are underway.

The size of the OxyR regulon varies greatly, ranging from dozens (most bacteria in which the subject has been studied) to over one hundred (<i>P. aeruginosa</i>) (31). Among the OxyR-binding sites predicted by regulatory sequence analysis tools (RSAT) (32), five verified members of the regulon are on the top, far above the remaining several based on RSAT weight values. This implies that the regulon of <i>S. oneidensis</i> OxyR is likely small. On the contrary, a microarray analysis of <i>S. oneidensis</i> cells in response to H<sub>2</sub>O<sub>2</sub>-induced oxidative stress has revealed that a vast number of genes are differentially transcribed (19). This indicates that oxidative stress imposes a global impact on gene expression mostly in an indirect manner. Most significantly, those associated with iron/heme biology except for Dps and thioredoxin/glutaredoxin systems involved in OxyR reduction, two groups of well-known OxyR-dependent genes in <i>E. coli</i> and many other bacteria, are not affected by the OxyR loss in <i>S. oneidensis</i> (19,30-31).

The physiological relevance as to why the OxyR regulon contracts so much is intriguing. It is tempting to suggest that this may be associated with redox-stratified niches where <i>Shewanella</i> thrive. As <i>Shewanella</i> respire an array of electron acceptors, mostly under microaerobic or anaerobic conditions, endogenous and exogenous ROS may not often amount to life-threatening levels. As such, these bacteria evolve a concise and sufficient protection system against oxidative stress in their living environments. In line with this, although small the regulon contains CcpA, cytochrome c551 peroxidase, which substantiates H<sub>2</sub>O<sub>2</sub> removal under anaerobic conditions but dispensable under aerobic conditions (34).

**EXPERIMENTAL PROCEDURES**

**Bacterial strains, plasmids and culture conditions** - All bacterial strains and plasmids used in this study can be found in Table 2. Information about all of the primers was available upon request. All chemicals were obtained from Sigma-Aldrich unless otherwise noted. <i>E. coli</i> and <i>S. oneidensis</i> were grown in Lysogeny broth (LB, Difco, Detroit, MI) under aerobic conditions at 37 and 30 °C for genetic manipulation. When necessary, the growth medium was supplemented with chemicals at the following concentrations: 2,6-diaminopimelic acid (DAP), 0.3 mM; ampicillin, 50 μg/ml; kanamycin, 50 μg/ml; and gentamycin, 15 μg/ml; streptomycin, 100 μg/ml; and catalase on plates, 2000 U/ml.

Growth in liquid media was monitored by recording values of optical density at 600 nM (OD<sub>600</sub>) as all strains used in this study were morphologically similar. Both LB and defined medium MS (18) were used for phenotypic
assays in this study and comparable results were obtained with respect to growth.

**DNA manipulations** - For inducible gene expression, the gene of interest was generated by PCR and introduced into the inducible plasmid pHGE-Ptac under the control of promoter Ptac (35). Take OxyR as an example: the resulting vector, pHGE-Ptac-oxyR was verified by sequencing, and transferred into the relevant strains via *E. coli* WM3064-mediated conjugation for chromosome integration as described before (28). To calibrate activities of Ptac integrated on the chromosome, Ptac was placed before the lacZ gene within integrative vector pHGEI01. The resulting vector, after verification by sequencing, was introduced into the *S. oneidensis* wild-type for chromosome integration. The same strategy was used to construct integrative lacZ reporter vectors carrying PkatB variants.

To construct *S. oneidensis* strains expressing OxyR<sup>C203S</sup> and OxyR<sup>L197P</sup> driven by IPTG-inducible Ptac, fragments of Ptac-oxyR<sup>T613C</sup> and Ptac-oxyR<sup>T607A</sup> were amplified from pHGE-Ptac-oxyR<sup>T613C</sup> and pHGE-Ptac-oxyR<sup>T607A</sup> respectively and cloned into pHGEI01. The resulting vector, after verification by sequencing, was introduced into the *S. oneidensis* wild-type for chromosome integration as described above.

**Analysis of gene expression** - Activity of target promoters was assessed using a single-copy integrative lacZ reporter system as described previously (28). Briefly, fragments of varying length (indicated in the relevant Figures) containing the sequence upstream of the target operon were amplified, cloned into the reporter vector pHGEI01, and verified by sequencing. The resultant vector in *E. coli* WM3064 was then transferred by conjugation into relevant *S. oneidensis* strains, in which it integrated into the chromosome and the antibiotic marker was removed subsequently (36). Cells grown to the mid-log phase under conditions specified in the text and/or figure legends were collected and β-galactosidase activity was determined by monitoring color development at 420 nm using a Synergy 2 Pro200 Multi-Detection Microplate Reader (Tecan) presented as Miller units.

For qRT-PCR, *S. oneidensis* cells were grown in LB with the required additives to the mid-log phase and collected by centrifugation, and RNA extraction was performed using the RNeasy minikit (Qiagen) as described before (37). RNA was quantified by using a NanoVue spectrophotometer (GE Healthcare). The analysis was carried out with an ABI7300 96-well qRT-PCR system (Applied Biosystems) as described previously (37). The expression of each gene was determined from three replicates in a single real-time qRT-PCR experiment. The Cycle threshold (C<sub>T</sub>) values for each gene of interest were averaged and normalized against the C<sub>T</sub> value of the 16s rRNA gene, whose abundance is relatively constant during the log phase. Relative abundance (RA) of each gene was presented.

**Droplet assays** - Droplet assays were employed to evaluate viability and growth inhibition on plates. Cells grown in LB to the mid-log phase were collected by centrifugation and adjusted to 10<sup>9</sup> cfu/ml (colony forming unit), which was set as the undiluted (dilution factor 0). Ten-fold series dilutions were prepared with fresh LB medium. Five microliters of each dilution was dropped onto LB plates containing agents such as catalase and/or IPTG when necessary. The plates were incubated for 24 h or longer in dark before being read. All experiments were repeated at least three times.
Disc diffusion assays - Disc diffusion assays were done similarly to those done previously (19). Briefly, overnight cultures were diluted into LB and grown to the mid-log phase (~0.6 of OD_600). One hundred microliters of cultures was spread onto an agar plate containing required chemicals. Paper discs (diameter, 8 mm) containing 10 μl of 10 M H_2O_2 were placed on top of the agar. The plates were incubated at 30 °C for 16 h prior to analysis. The diameters of the zones of clearing (zones of inhibition, in millimeters) generated by the peroxides were measured. All assays were done in triplicate using independent cultures, and the resulting zones of inhibition were averaged.

Analysis of catalase - To assess catalase levels, *S. oneidensis* cells grown in LB the mid-log phase were incubated with 0.2 mM H_2O_2 for 30 min and then collected by centrifugation, and disrupted by French pressure cell treatment. Throughout this study, the protein concentration of the resulting cell lysates was determined using a Bradford assay with BSA as a standard (Bio-Rad). Aliquots of cell lysates containing the same amount of protein were subjected to 10% nondenaturing polyacrylamide gel electrophoresis (PAGE). Catalases were detected by using the corresponding activity staining methods (38).

Activity of catalase was also assayed in a more quantitative approach as described previously (19). Briefly, mid-log-phase cells in liquid medium were collected, washed twice in 50 mM KH_2PO_4 buffer (pH 7.0), and resuspended in the same buffer, and then disrupted by sonication. Ten microliter of cell extracts containing 40 ng/μl protein was added to 90 μl KH_2PO_4 and 100 μl 20 mM H_2O_2 in a 200-μl-volume. Decomposition of H_2O_2 was measured at 240 nm with absorbance readings taken at 15-s time intervals for a total time of 3.5 min in a Tecan M200 Pro microplate reader. The unit of activity of each sample is expressed as μmol H_2O_2 decomposed per min and per mg of protein (μmol · min^{-1} · mg^{-1}). Each sample was tested in quadruplicate for each strain assayed.

Site-directed mutagenesis - The coding sequence of *S. oneidensis* oxyR was amplified by PCR and cloned in pET28 for expressing recombinant proteins with N-terminal six-histidine tag. The resulting pET28-oxyR, after sequencing verification, was used as the template for site-directed mutagenesis to express OxyR^{C203S} and OxyR^{L197P} with a QuikChange II XL site-directed mutagenesis kit (Stratagene) as described previously (39). The resulting pET28-oxyR^{T607A} and pET28-oxyR^{T613C} was verified by sequencing.

Expression and purification of OxyR variants. Full-length - *S. oneidensis* OxyR^{WT}, OxyR^{C203S}, and OxyR^{L197P} were purified as His-tagged soluble proteins. *E. coli* BL21(DE3) strains carrying pET28-oxyR, pET28-oxyR^{T607A}, or pET28-oxyR^{T613C} grown in LB to the mid-log phase were induced with 0.2 mM IPTG at 25°C for 6 h to produce high levels of His_6-OxyR variants. His_6-OxyR variants were purified from crude cell lysates by French pressure cell treatment over a nickel-ion affinity column (GE health). After removal of contaminant proteins with washing buffer containing 20 mM imidazole, the His-tagged OxyR variants were eluted in elution buffer containing 100 mM imidazole. The proteins were concentrated by ultrafiltration (10-kDa cutoff), exchanged into 20 mM Tris-HCl (pH 8.0) containing 150 mM NaCl, and further purified by gel filtration using a Superdex 200 column (Pharmacia) run on an Äkta fast protein liquid chromatography (FPLC) system (Pharmacia). The peak fractions were
analyzed by 12% SDS-PAGE, followed by staining with Coomassie brilliant blue R250. Identification of purified proteins was confirmed with MS/MS analysis as described before (40).

**Chemical cross-linking of OxyR** - Chemical cross-linking with ethylene glycol bis-succinimidylsuccinate (Pierce Chemical Co., Rockford, Ill.) was used to examine the solution structure of OxyR. Cross-linking was initiated by the addition of ethylene glycol-bis succinimidylsuccinate in dimethyl sulfoxide to a final concentration of 10 mM, with 2.5 μg of purified His<sub>6</sub>-OxyR in a total volume of 20 μl. The reaction mixtures were incubated at room temperature for 30 min, quenched with 50 mM glycine, and analyzed by 12% SDS-PAGE.

**DNA binding analyses** - To test interaction between OxyR and promoter regions of its target genes, electrophoretic mobility shift assays (EMSA) were conducted as previously described (41). DNA binding assays and conditions were similar to those previously reported, although a fluorescent label was used in place of radioactivity to detect the promoter fragment. DNA probes covering the predicted OxyR binding sites were obtained by PCR, during which the double-stranded product was labelled with digoxigenin-ddUTP (Roche diagnostics). The digoxigenin-labeled DNA probes were mixed with serial dilutions of purified OxyR of varying concentrations in binding buffer (4 mM Tris-HCl [pH 8.0], 40 mM NaCl, 4 mM MgCl<sub>2</sub>, 4% glycerol) containing 0.75 μg of poly(dl-dC) at room temperature for 15 min. The DNA/protein mixtures were loaded on 7% native polyacrylamide gels for electrophoretic separation and the resulting gel visualized with the UVP image system.

Fluorescence anisotropy was chosen as a method of DNA binding analysis in equilibrium (42). DNA oligonucleotides 5′-end labeled with 6-carboxyfluorescein (FAM) were ordered from Sangon (Shanghai) and resuspended in annealing buffer (10 mM Tris, pH 7.5, 50 mM NaCl, 0.05 mM EDTA). OxyR of increasing protein titrations was incubated with a solution containing 10 nM 6-FAM-labeled DNA, 100 nM competitive nonspecific DNA, 100 mg/liter bovine serum albumin (BSA), 100 mM Bis-Tris (pH 6.5), 150 mM NaCl, and 5% glycerol and added to a constant volume of 25 μl in a 384-well black assay plate (Costar). Fluorescence anisotropy of the fluorescein-labeled DNA was observed via excitation at 485 nm and emission at 520 nm, using a plate reader (M200Pro, Tecan). Measurements were made in triplicate, and reported values are the averages for three separate triplicate runs. Data were plotted as average fluorescence anisotropy values as a function of protein concentration by using Graphpad Prism v7. The $K_d$ (dissociation constant) was calculated based on the equation generated by the best-fit curve using a single-ligand binding equation: $r = ([P] \times \Delta r)/(K_d + [P])$, where $r$ is the anisotropy value, $\Delta r$ is the change in anisotropy, [P] is the concentration of added protein, and $K_d$ is the dissociation constant of the protein with the DNA probe. Each OxyR variant was analyzed at each experiment by three individual replicates.

**5′-RACE analysis of transcripts** - The transcriptional start site of the *katB* gene of *S. oneidensis* was determined using rapid amplification of cDNA ends (5′-RACE) kit (Invitrogen) as recently described (43). RNAs from *S. oneidensis* cells of the mid-log phase was extracted and quantified as described above. Reverse transcription was conducted on preprocessed RNAs without 5′-phosphates
followed by two rounds of nested PCRs. The PCR products were subjected to agarose gel separation, with purification of the 5’-RACE products, and inserted into the pMD19-T vector (TaKaRa) for direct DNA sequencing. The first DNA base adjacent to the 5’-RACE adaptor was regarded as the transcription start site.

**DNase I footprinting** - DNase I footprinting analysis was carried out as described elsewhere (44). DNA fragment of 152 bp with the predicted OxyR-binding site were generated by PCR using 6-FAM primers labeled at the 5’-end. Binding reactions for DNase I footprinting were conducted as for the EMSAs. The digested DNA was subjected to DNA sequencing and analyzed with Peak Scanner software (Applied Biosystems).

**Other analyses** - AlignACE (45) and MDScan (46) were used to identify OxyR-binding motifs in the putative promoter regions of genes. Sequence logos were generated using WebLogo (47). Student’s t test was performed for pairwise comparisons with statistical significance set at the 0.05 confidence level. Values were presented as means ± standard error of the mean (SEM).
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Author contributions: HG conceived the idea for the project and coordinated the study, and wrote the manuscript. FW and LK performed the experiments and analyzed the data, and wrote the manuscript. All authors reviewed and approved the final version of the manuscript.

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**FIGURE LEGENDS**

**FIGURE 1.** Characteristics of purified *S. oneidensis* OxyR protein. *A*, SDS-PAGE analysis of Purified recombinant OxyR protein after gel exclusion chromatography. *B*, SDS-PAGE analysis of OxyR treated with dithiothreitol (DTT). The reaction mixtures consisted of 5 μM OxyR protein and DTT at indicated concentrations. R and O represent reduced and oxidized forms of the protein. *C*, Chemical cross-linking analyses of the purified OxyR protein. The ethylene glycol bis-succinimidylsuccinate (EGS) cross-linking reagent was added at the concentrations shown at the top. In all panels, M represents protein standard marker.

**FIGURE 2.** Physiological impacts of reduced and oxidized *S. oneidensis* OxyR proteins. *A*, Droplet assays for viability and growth assessment. Cultures of indicated strains prepared to contain approximately 10^9 cfu/ml were regarded as the undiluted (dilution factor, 0), which were subjected to 10-fold series dilution. Five microliters of each dilution was dropped on LB plates containing IPTG at indicated concentrations. Results were recorded after 24 h incubation. Expression of OxyR variants was driven by IPTG inducible P\(_{\text{tac}}\). *B*, H\(_2\)O\(_2\) susceptibility by disc diffusion assay. One hundred microliters of cultures was spread onto an agar plate containing 0.02 mM IPTG. Paper discs (diameter, 8 mm) containing 10 μl of 10 M H\(_2\)O\(_2\) were placed on top of the agar. The plates were incubated at 30 °C for 16 h prior to analysis. The diameters of the zones of clearing (halo, in millimeters) generated by the peroxides were measured. *C*, Catalase detected by staining and activity assay. Cells of the mid-log phase either directly used (non-treated) or incubated with 0.2 mM H\(_2\)O\(_2\) for 30 min (treated) were used for the assay. Cell lysates containing the same amount of protein were subjected to 10% nondenaturing PAGE (upper panel) and activity assay (lower panel). Staining was...
performed as described in experimental procedures. For catalase activity assay, Decomposition of H$_2$O$_2$ was measured at 240 nm with absorbance readings taken at 15-s time intervals for a total time of 3.5 min. The unit of activity of each sample is expressed as catalase unit (µmol H$_2$O$_2$ decomposed per min and per mg of protein).

Analysis of ahpC and katB transcripts by qRT-PCR. Cells of the mid-log phase incubated with 0.2 mM H$_2$O$_2$ for 2 min were used for total RNA extraction. The Cycle threshold ($C_T$) values were averaged and normalized against the $C_T$ value of the 16s rRNA gene, RTA, relative transcript abundance. In all panels, experiments were performed at least three times, with the average ± error bars representing standard errors or representative results being presented.

FIGURE 3. Characterization of the *S. oneidensis* katB promoter region. A, The promoter region of the katB gene. Binding site for OxyR is in red and underlined (discuss later in Fig. 6B) and transcription starting site (TSS) is in green and underlined. The number of nucleotides is relative to the translational starting code. B, Determination of the katB transcriptional start site using 5’-RACE. The result of direct DNA sequencing the 5’-RACE product of the katB gene was shown. C, Deletion mapping of the katB regulatory region. Transcriptional fusion constructs are diagramed; coordinates indicate the extent of the regulatory katB region cloned in front of the lacZ reporter gene. The plasmids carrying the constructs were introduced into the relevant strains and integrated on the chromosome. After the antibiotic marker removal, promoter activity was measured by β-galactosidase assay and presented as Miller Units. Experiments were performed at least three times, with the average ± error bars representing standard errors being presented.

FIGURE 4. Both reduced and oxidized *S. oneidensis* OxyR proteins interact with the katB promoter region. A, *In vitro* interaction of His-tagged OxyR variants and the katB promoter sequence revealed by using EMSA. The Digoxigenin-labeled DNA probes were prepared by PCR. The EMSA assay was performed with 1 µM probes and various amounts of proteins as indicated. Non-specific competitor DNA (2 µM poly dI·dC) was included. B, DNase I footprinting analysis of OxyR variants. OxyR variants at 2 µM were used for binding to the 6-FAM labelled katB promoter fragment. The regions protected by OxyR variants are indicated by a black dotted box and given below for clarity.

FIGURE 5. *S. oneidensis* OxyR$_{oxi}$ (OxyR$^{L197P}$) outcompetes OxyR$_{red}$ (OxyR$^{C203S}$) for binding to the katB promoter. A, fluorescence anisotropy change upon titration of a limiting concentration of FAM-labeled 60-bp katB oligonucleotides (10 nM) with indicated OxyR variants. Data were plotted as fluorescence anisotropy change values in millianisotropy units (mA) as a function of protein concentration by using Graphpad Prism v7 and fit to a model describing a 1:1 protein tetramer, and lines represent simulated curves produced from the average. B, Impacts of OxyR variants at varying levels on growth of the wild-type with droplet assays. Production of OxyR variants was driven by P$_{tac}$ from a single copy on the chromosome. Production level in the presence of 0.02 mM IPTG was equivalent to that of from the native oxyR promoter. Catalase was added to
differentiate defects in viability and in growth. Experiments were performed at least three times, with the average ± error bars representing standard errors being presented or representative results being presented.

FIGURE 6. **Binding motifs of S. oneidensis OxyR.** A, *In vitro* interaction of His-tagged OxyR<sub>L197P</sub> and the various promoter sequences revealed by using EMSA. Experiments were performed the same as Fig. 4A. B, Predicted OxyR-binding motifs in *S. oneidensis* based on the verified promoter sequences. The motifs of 13-bp and 29-bp containing palindromic sequences predicted by AlignACE and MEME was marked with black and blue dash lines, respectively. Tetranucleotide sequences were underlined based on *E. coli* OxyR consensus. C, Mutational analysis of the *katB* regulatory region. Transcriptional fusion constructs are diagramed as in Fig. 4C. In P<sub>katB</sub>, the motif of 13-bp is in blue and extended to form the 29-bp motif, the additional nucleotides are in red, and tetranucleotide sequences were underlined. In P<sub>katB</sub> mutants, dot represents the corresponding nucleotide deleted and nucleotides underlined and in bold were mutated. Promoter activity was measured by β-galactosidase assay and presented as Miller Units. Experiments were performed at least three times, with the average ± error bars representing standard errors being presented.
Table 1. Top OxyR-binding sites predicted to be in *S. oneidensis*

| Locus   | Gene | Strand | Location | Binding sequence                                      | Weight | Predicted function                                      |
|---------|------|--------|----------|-------------------------------------------------------|--------|--------------------------------------------------------|
| SO_1158 | *dps*| D      | 101-63   | AATCTAAATAACAGATTGATAAAATCTATTTTAACCGCT              | 28.1   | Dps family protein                                     |
| SO_0958 | *ahpC*| D      | 140-102  | ATTCGACAAAAACCAGATTGAAACAATAGTTTTTATTGCCGTT          | 27.8   | Alkyl hydroperoxide reductase, C subunit               |
| SO_0725 | *katG-1*| D      | 215-177  | AATCTGGTTTCGCGATTCCACCATTGGTTTTAATCGTT               | 27.7   | Catalase/peroxidase HPI                                 |
| SO_2178 | *ccpA*| D      | 115-77   | AATCCACAACAGCGATTGAGACCATCGGTATTTAATCGTT             | 27     | Cytochrome c551 peroxidase                              |
| SO_1070 | *katB*| D      | 171-133  | ATCTTTAAGTAGATTGTAAGAATCTACTTATATCGTT                | 26.6   | Catalase                                              |
| SO_r012 | *rrsD*| R      | 144-106  | CATCTGGTTAGCAATTGAAACATTTGATTTAATCGTT                | 14.4   | 16S ribosomal RNA                                      |
| SO_2530 | *def*| D      | 119-81   | AATCTAAAAACCGATTGTAATTTATTTATTTAATCGTT              | 14     | Polypeptide deformylase                                 |
| SO_3490 |       | D      | 137-99   | TATCCCTAAACACAAATGACAGCTATATTATATTACCCTGTC          | 12     | Protein of unknown function DUF88                     |
| SO_2842 |       | D      | 118-80   | AGTTGAAAATAGCGTTTGCTAATAATCGTTTTTGTGAT              | 9.5    | Peptidase, M23/M37 family                              |
| SO_1984 |       | D      | 187-149  | AATTGAGATAATCCATTGACATTGATAACGTTCT ACT              | 7.9    | Hypothetical protein                                   |
| SO_0921 |       | R      | 63-25    | AAACCTTATTCAAGCCTAGCAGACTAAGTTTAATCGTA              | 7.8    | Hypothetical protein                                   |

*Sites shared by two ORFs: SO_2530-SO_2532 (O-6-methylguanine-DNA-alkyltransferase AdaA), SO_3489 (diguanylate cyclase)-SO_3490, SO_0920 (acteyltransferase GNAT family)-SO_0921.*
### Table 2. Strains and plasmids used in this study

| Strain or plasmid | Description | Reference or source |
|-------------------|-------------|---------------------|
| **E. coli strains** |             |                     |
| DH5α              | Host for cloning | Lab stock          |
| BL21              | Recombinant protein expression host strain | Novagen         |
| WM3064            | ΔdapA, donor strain for conjugation | W. Metcalf, UIUC |
| **S. oneidensis strains** |             |                     |
| MR-1              | Wild type | Lab stock          |
| HG1070            | ΔkatB derived from MR-1 | (19)         |
| HG1328            | ΔoxyR derived from MR-1 | (19)         |
| HG-OxyR<sup>1977F</sup> | MR-1 carrying integrated P<sub>tac</sub>-oxyR<sup>T613C</sup> | This study |
| HG-OxyR<sup>1977F</sup> | MR-1 carrying integrated P<sub>tac</sub>-oxyR<sup>T607A</sup> | This study |
| **Plasmid**       |             |                     |
| pHGE-Ptac         | Km<sup>r</sup>, IPTG-inducible expression vector | (35)       |
| pHGEI01           | Km<sup>r</sup>, integrative lacZ reporter vector | (28)       |
| pBBR-Cre          | Sp<sup>r</sup>, helper plasmid for antibiotic cassette removal | (36)       |
| pET-28a           | Recombinant protein expression vector | Novagen |
| pHGE-Ptac-oxyR<sup>1977F</sup> | P<sub>tac</sub>-oxyR<sup>T613C</sup> within pHGE-Ptac | This study |
| pHGE-Ptac-oxyR<sup>1977F</sup> | P<sub>tac</sub>-oxyR<sup>T607A</sup> within pHGE-Ptac | This study |
| pHGEI01-PkatB-v    | All PkatB variants-lacZ fusion within pHGEI01 | This study |
| pET-oxyR<sup>T613C</sup> | oxyR<sup>T613C</sup> within pET-28a | This study |
| pET-oxyR<sup>T607A</sup> | oxyR<sup>T607A</sup> within pET-28a | This study |
|                  | OxyR<sup>WT</sup> (nM) | OxyR<sup>C203S</sup> (nM) | OxyR<sup>L197P</sup> (nM) |
|------------------|------------------------|---------------------------|--------------------------|
|                  |- 10 50 250              | - 2.5 12.5 62.5           | - 10 50 250              |

**OxyR<sup>WT</sup>**

**OxyR<sup>C203S</sup>**

**OxyR<sup>L197P</sup>**

---

**probe** → shift → super-shift →

---

**katB**

16s rRNA gene

---

**No OxyR**

**OxyR<sup>WT</sup>**

**OxyR<sup>C203S</sup>**

**OxyR<sup>L197P</sup>**

---

**OxyR<sup>WT</sup>:** TAATCTTTAAAGTAGATTGTAAGAATCTACTTTAATCGTTTTATTGGTTTACAT

**OxyR<sup>C203S</sup>:** AATCTTTAAAGTAGATTGTAAGAATCTACTTTAATCGTTTTATTGGTTTACAT

**OxyR<sup>L197P</sup>:** TAATCTTTAAAGTAGATTGTAAGAATCTACTTTAATCGTTTTATTGGTTTACAT
EcOxyR consensus:       ATAGntnnnanCTAT-N7-ATAGntnnnanCTAT

B
 probe  A
  0   10   50 ...
100
200
300
400
500
600
700
800
WT WT M1 M2 M3 M4 M5 M6
Miller Units
A B H2O2:   H2O2: +

C

P_{katB}^{-}       AATCTTTAAAGT\textcolor{red}{AGAToggATAGAATCTACTTTTAACTCGTT}
P_{katB}^{-}M1:    AATCTTTAAAAGTA\ldots\ldots\ldots TACTTTAATCGTT
P_{katB}^{-}M2:    AATCTTTAAAAGT\textcolor{red}{AGAToggATAGAATCTACTTTTAACTCGTT}
P_{katB}^{-}M3:    AATC\ldots\ldots\ldots GTAGAT\textcolor{red}{AGAToggATAGAATCTACTTTTAACTCGTT}
P_{katB}^{-}M4:    AATCTTTTTCTGAGAT\textcolor{red}{AGAToggATAGAATCTACTTTTAACTCGTT}
P_{katB}^{-}M5:    AATCTTTAAAGT\textcolor{red}{AGAToggATAGAATCTACTTTTAACTCGTT}
P_{katB}^{-}M6:    AATCTTTAAAAGT\textcolor{red}{AGAToggATAGAATCTACTTTTAACTCGTT}

ΔoxyR
Defining the binding determinants of Shewanella oneidensis OxyR: implications for the link between the contracted OxyR regulon and adaptation
Fen Wan, Linggen Kong and Haichun Gao

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