Impaired cell envelope resulting from \textit{arcA} mutation largely accounts for enhanced sensitivity to hydrogen peroxide in \textit{Shewanella oneidensis}

Fen Wan, Yinting Mao, Yangyang Dong, Lili Ju, Genfu Wu & Haichun Gao

Oxidative stress is one of the major challenges that \textit{Shewanella} encounter routinely because they thrive in redox-stratified environments prone to reactive oxygen species (ROS) formation, letting alone that ROS can be generated endogenously. As respiration is the predominant process for endogenous ROS, regulators mediating respiration have been demonstrated and/or implicated to play a role in oxidative stress response. In our efforts to unveil the involvement of global regulators for respiration in the oxidative stress response, we found that loss of the Arc system increases \textit{S. oneidensis} sensitivity to H$_2$O$_2$ whereas neither Fnr nor Crp has a significant role. A comparison of transcriptomic profiles of the wild-type and its isogenic \textit{arcA} mutant revealed that the OxyR regulon is independent of the Arc system. We then provided evidence that the enhanced H$_2$O$_2$ sensitivity of the \textit{arcA} mutant is due to an increased H$_2$O$_2$ uptake rate, a result of a cell envelope defect. Although one of three proteases of the ArcA regulon when in excess is partially accountable for the envelope defect, the major contributors remain elusive. Overall, our data indicate that the Arc system influences the bacterial cell envelope biosynthesis, a physiological aspect that has not been associated with the regulator before.

Microorganisms live in environments abundant with various perturbations and therefore develop general and specific stress responses. Oxidative stress, caused by reactive oxygen species (ROS), is arguably the most unavoidable to cells in aerobic environments because cells on their own inevitably and continuously produce ROS from autooxidation of components of the respiratory chain (1). ROS damages a variety of cellular macromolecules, such as DNA, RNA, proteins, and lipids. To keep the concentration of ROS at an acceptable level and to repair oxidative damages, cells evoke a cellular mechanism responding to oxidative stresses that permits survival. In bacteria, the primary defense comprises superoxide dismutase, catalase, and peroxidase that directly remove excess ROS and glutathione/glutaredoxin/thioredoxin that help maintain an intracellular reducing environment thus limiting ROS damages (2). In parallel, bacterial cells are equipped with a repairing system, consisting of endonuclease, proteolytic, and lipolytic enzymes, which functions as the secondary defense by removing damaged cellular components.

In bacteria, the oxidative stress response is predominantly mediated by four transcriptional regulators, OxyR, SoxRS, PerR, and OhrR (1,3). The OxyR system, widely found in Gram-negative bacteria, responds to hydrogen peroxide (H$_2$O$_2$) mostly as an activator, whereas the less omnipresent SoxRS system is activated by redox-active compounds to prevent damages on macromolecules from superoxides (4). In many Gram-positive bacteria, PerR takes place of OxyR responding to H$_2$O$_2$ as a repressor. OhrR,
conserved among Gram-negative and -positive bacteria, is specific for responding to organic peroxide (OP) (3). In addition, there exists the extensive connectivity between the regulons specific to the oxidative stress response and other regulatory systems. For instance, RpoS (σ54) is important for expression of many genes that are induced under a variety of stresses and Fur, the principal regulator of iron homeostasis, influences transcription of some members of the ROS-specific regulons by interacting with their Fur-binding sequences (5). Moreover, two redox-sensing global regulatory systems mediating the transition from aerobic to anaerobic metabolism, Fnr (fumarate and nitrate reduction regulator) and Arc (aerobic respiration control) two-component system, have been implicated to have an important role in the resistance to ROS-induced damage (6–12).

*Shewanella*, facultative Gram-negative anaerobes renowned for their remarkable anaerobic respiratory abilities, become a research model for investigating redox transformations of a variety of inorganic and organic chemicals, with *S. oneidensis* as most intensively studied (13). Compared to *Escherichia coli*, *S. oneidensis* is hypersensitive to H₂O₂ and to all wavelengths of solar UV, UV and ionizing radiation, a process linked to ROS generation (14). Surprisingly, despite intense interest in the mechanisms by which *Shewanella* copes with environmental stresses (15), how this group of bacteria responding to oxidative stresses has not been investigated until recently (16–17). Like most other Gram-negative bacteria, *S. oneidensis* uses OxyR and OhrR as the master regulators to mediate its response to H₂O₂ and OPs respectively, but lacks an analogue of SoxR (16–17). The OxyR and OhrR regulons appear to be functionally intertwined as both OxyR and OhrR systems can sense and respond to H₂O₂ and OP agents (17). Interestingly, while *S. oneidensis* is fully equipped with a whole package of enzymes to scavenge ROS, the genes encoding proteins for the secondary defense are either missing or unresponsive to the stress (16). Moreover, OxyR plays an important role in phase induction and biofilm formation (18). Interestingly, while *S. oneidensis* is fully equipped with a whole package of enzymes to scavenge ROS, the genes encoding proteins for the secondary defense are either missing or unresponsive to the stress (16). Moreover, OxyR plays an important role in phase induction and biofilm formation (18).

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Given that oxygen availability is generally intertwined with oxidative stress, in this study we attempted to determine the involvement of *S. oneidensis* Fnr, Crp, and Arc in oxidative stress response. We showed that loss of the Arc system elevates sensitivity to H₂O₂ whereas neither Fnr nor Crp has a significant role in response to the agent. A comparison of the transcriptomic changes elicited by H₂O₂ between the wild type and *arcA*-deficient strains demonstrated that the Arc system is not an important player in co-regulating genes belonging to the OxyR regulon. The enhanced H₂O₂ sensitivity of the *arcA* mutant was then linked to an increased H₂O₂ uptake rate, a result of a cell envelope defect. We then showed that this defect is partially attributable to overproduction of SO1915, one of three proteases of the ArcA regulon. Moreover, it seems that the impaired envelope resulting from the *arcA* mutation is rather complex, unlikely depending on a single gene. The present study for the first time links the Arc system with the biosynthesis of bacterial cell envelope.

**Methods**

**Bacterial strains, plasmids, and culture conditions.** A list of all bacterial strains and plasmids used in this study is given in Table 1. Information for primers used in this study was available upon request. *E. coli* and *S. oneidensis* strains under aerobic conditions were grown in Lysogeny Broth (LB, Difco, Detroit, MI) medium (31), which was modified to contain tryptone (10 g/L), yeast extract (5 g/L), and NaCl (5 g/L), at 37 °C and 30 °C for genetic manipulation. When needed, the growth medium was supplemented with chemicals at the following concentrations: 2, 6-diaminopimelic acid (DAP), 0.3 mM; ampicillin sodium, 50 μg/ml; and gentamycin sulfate, 15 μg/ml.

**In-frame mutant construction and complementation.** In-frame deletion strains for *S. oneidensis* were constructed using the att-based fusion PCR method as described previously (32). In brief, two fragments flanking the target gene were amplified by PCR with the gene specific primers, which were joined by the second round of PCR. The fusion fragments were introduced into plasmid pHGM01 by using Gateway BP clonase II enzyme mix (Invitrogen) according to the manufacturer’s instruction, resulting in mutagenesis vectors in *E. coli* WM3064, which were subsequently transferred into relevant *S. oneidensis* strains via conjugation. Integration of the mutagenesis constructs into the chromosome was selected by the second round of PCR. The fusion fragments were then linked to an increased H₂O₂ uptake rate, a result of a cell envelope defect. We then showed that this defect is partially attributable to overproduction of SO1915, one of three proteases of the ArcA regulon. Moreover, it seems that the impaired envelope resulting from the *arcA* mutation is rather complex, unlikely depending on a single gene. The present study for the first time links the Arc system with the biosynthesis of bacterial cell envelope.

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Physiological characterization of *S. oneidensis* in response to H$_2$O$_2$ and SDS. Growth of *S. oneidensis* strains generated in this study was measured by recording the optical density at 600 nm (OD$_{600}$) values in triplicate with the wild-type as the control in M1 defined medium containing 0.02% (w/v) of vitamin-free Casamino Acids and 15 mM lactate as described previously (29). Impacts of H$_2$O$_2$ on *S. oneidensis* strains were assessed by three approaches. First, minimum inhibitory concentrations (MIC) of H$_2$O$_2$ and SDS were determined in LB as described previously (16). Second, disk diffusion assays were carried out. Briefly, cultures of mid-log phase (~0.4 of OD$_{600}$, same afterwards) were properly diluted and spread onto fresh LB plates (200 μl of culture; approximately 10$^6$ colony forming units (cfu). Paper discs of 6 mm in diameter loaded with 10 μl H$_2$O$_2$ of 5 M were placed onto the bacterial lawn and plates were incubated at 30 °C for 16 h. Third, survival of *S. oneidensis* strains was assayed. H$_2$O$_2$ was added to the mid-log phase cultures to a final concentration of 1.0 mM and samples were taken at 5 and 30 min. The cultures were serially diluted with fresh LB and plated onto LB plates. Plates from dilutions that gave 100 to 250 cfu were counted.

To measure H$_2$O$_2$ consumption, mid-log phase cells were collected, washed twice in 50 mM NaHPO$_4$ buffer (pH 7.0) and resuspended in the same buffer to an OD$_{600}$ of 0.1. H$_2$O$_2$ was added to a final concentration of 0.5 mM and the cells were incubated at 30 °C. Aliquots were assayed for remaining H$_2$O$_2$ 1, 5, and 10 min after the treatment began using the FOX method (34).

Table 1. Strains and plasmids used in this study.
The susceptibility of *S. oneidensis* strains to SDS was assessed by a drop plate assay. Cells at mid-log phase were adjusted to approximately 10⁶ CFU/ml with fresh LB medium, followed by 6 10-fold serial dilutions. Five microliters of each dilution was spotted onto LB plates containing SDS. The plates were incubated for 24 h or longer before being read. The cell envelope defect was also assessed by adding SDS into mid-log phase cultures and monitoring optical density reduction.

**Microarray analysis.** Microarray analysis was performed essentially the same as described previously (16). In brief, for each strain under aerobic conditions, 20ml of LB in a 100 ml shake flask was inoculated with fresh overnight culture to OD600 of 0.01 and shaken on a rotary platform (250 rpm) until the mid-log phase. Cultures were divided into two parts; one was used as the untreated control and the other was applied to H₂O₂ at a final concentration of 0.2 mM for a treatment of 5 min. All cultures were centrifuged at 14000 rpm for 30 s at room temperature and the pellets were frozen immediately in liquid nitrogen and stored at −80°C. In total, four biological replicas under each condition were prepared. DNA microarrays, total RNA extraction, cDNA labeling, hybridization, slide scanning, and data analysis were described previously (29,35). Refer to NCBI GEO accession number GSE31053 for raw microarray data.

**Electrophoretic motility shift assay (EMSA).** Expression and purification of His-tagged *S. oneidensis* ArcA has been described before (29,36). Phosphorylation of purified ArcA was performed in buffer containing 100 mM Tris/HCl (pH 7.0), 10 mM MgCl₂, 125 mM KCl, 50 mM dithioll carbamoyl phosphate for 60 minutes at room temperature. The probes used for EMSA were prepared by PCR with ³²P end-labeled primers (29). The binding reaction was performed with ~25–50 fmol (~2–5 nM) labeled probes and various amounts of protein in 12 μl binding buffer containing 100 mM Tris/HCl (pH 7.4), 20 mM KCl, 10 mM MgCl₂, 2 mM DTT, 0.2 μg/μl poly(dI·dC), and 10% glycerol at 15°C for 60 minutes and resolved on pre-run 4.8% polyacrylamide native gels. Band shifts were visualized by autoradiography.

**Expression analysis.** β-Galactosidase activity assay was performed to determine gene expression. DNA fragments of ~400 bp covering sequences upstream of target genes were amplified and placed in front of the full-length *E. coli lacZ* gene on plasmid pHGEI01 (37). The resulting vector was transformed into *E. coli* WM3064, verified by sequencing and then transferred into *S. oneidensis*. Cells of mid-log phase prepared as for the microarray analysis were harvested by centrifugation, washed with phosphate-buffered saline (PBS, pH 7.0), and lysed with the lysis buffer (0.25 M Tris/HCl, pH 7.5, 0.5% Triton X-100). The resulting soluble protein was collected after centrifugation and used for enzyme assay by adding the aliquot of the o-nitrophenyl-β-D-galactopyranoside (ONPG) (4 mg/ml). The protein concentration of the cell lysates was determined using a Bradford assay with BSA as a standard (Bio-Rad). β-galactosidase activity were determined by monitoring color development at 420 nm using a Synergy 2 Pro200 Multi-Detection Microplate Reader (Tecan), presented as Miller units.

**In vivo diffusion of H₂O₂.** Five ml mid-log cells were pelleted by centrifugation for 5 min at 4500 rpm, suspended in 1 ml of 50 mM sodium phosphate buffer, pH 7.2. Aliquots of the suspension in a 200 μl volume were incubated with 0.5 mM H₂O₂ for 5 min and then vacuum-filtered using polycarbonate filters (Millipore). The flow-through, taken as extracellular portion, was collected and cells on the filter were suspended with 2 ml of the same suspension buffer and disrupted by French press, which was taken as intracellular portion. Both the extracellular and intracellular fractions were incubated separately with 2 μM horseradish peroxidase and 2 μM scopoletin and the resultant fluorescence was measured at 350 nm (excitation) and 460 nm (emission) using a Synergy 2 Pro200 Multi-Detection Microplate Reader (Tecan) as described elsewhere (39). The background fluorescence from a control cell suspension not exposed to H₂O₂ was subtracted and resulting values were normalized by protein concentrations. H₂O₂ uptake was determined as the extracellular/intracellular fluorescence ratio.

**Other analyses.** Protein subcellular localization prediction was carried out with PSORTb (40). Genome screening for ArcA-binding sites based on established weight matrices from *S. oneidensis* was performed using regulatory sequence analysis tools (RSAT) (41). Experimental values were subjected to statistical analyses and presented as means ± SD (standard deviation). Student’s *t*-test was performed for pairwise comparisons of groups.

**Results.** *S. oneidensis arcA* mutant shows an enhanced sensitivity to H₂O₂. As a starting point for this work, we examined physiological impact of H₂O₂ on the *S. oneidensis* arcA, crp, and fnr in-frame deletion strains. The MIC values for the wild-type, ΔarcA, Δcrp, and Δfnr strains were 1.25, 0.625, 1.25, and 1.25 mM at 30°C, respectively. Because of differences in growth rates (20), incubation times for tested strains were adjusted accordingly, relative to 16 h of the wild-type. By using a disk diffusion test, susceptibility of these *S. oneidensis* strains to H₂O₂ was measured (Fig. 1A). Instead of the low density of cells used in determining MIC, H₂O₂ discs were applied to a bacterial cell lawn. It was found that the *fnr* deletion strain was indistinguishable from the wild type. In contrast, the ΔarcA strain was significantly
more sensitive to H$_2$O$_2$, with a clear zone approximately 1.4-fold greater than that of the wild-type. In the case of the crp gene, it appeared that its loss resulted in a slightly increase in susceptibility. In addition, the viability of mid-log phase cells in the presence of 1 mM of H$_2$O$_2$ was estimated by counting cfu at 5 and 30 min postexposure (Fig. 1B). Similar to results from the disk diffusion test, the treatment did not reveal any significant difference in viability between the wild-type and Δfnr strains. Loss of either the crp or arcA gene affected the survival when compared to the wild-type. Apparently, the ΔarcA strain was substantially more vulnerable to H$_2$O$_2$ than the wild-type whereas the Δcrp strain was impaired marginally. Given that Crp is a global regulator involved in many metabolic processes under both aerobic and anaerobic conditions (22–25), we speculate that the slightly increased susceptibility and reduced viability upon the H$_2$O$_2$ treatment are likely due to its pleiotropic effect on the physiology. On the contrary, reasons underlying increased sensitivity of S. oneidensis to H$_2$O$_2$, resulting from the loss of the Arc system deserve further investigation, as this system has been implicated in the oxidative stress in many bacteria but the proposed mechanisms are diverse (6,9–10,42).

OxyR functions normally in the ΔarcA strain. To screen for candidate genes for the observed phenotype caused by the loss of ArcA, a microarray analysis was conducted to illustrate the transcriptomic differences elicited by the arcA mutation. We sampled mid-log-phase cells 5 min after the addition of 0.2 mM H$_2$O$_2$ in order to examine the transcriptional response. This experimental setting allows us to catch the most drastic changes at the transcriptional level without significantly killing cells (16,35). From collected samples, mRNAs were extracted, processed, and applied to microarray chips for hybridization (29,35). The statistical analysis revealed 989 genes whose expression was significantly altered, representing approximately 21% of the total ORFs spotted. Compared to the data from the parental wild-type strain under the same condition, only 20 of these 989 genes displayed an opposite transcription pattern

Figure 1. Loss of ArcA increases the H$_2$O$_2$ sensitivity of S. oneidensis. ΔoxyR was included for comparison, which had reduced sensitivity because derepression of the predominant catalase KatB. ΔarcA$^C$ and ΔoxyR$^C$ represents these mutants carrying a copy of the corresponding missing gene for complementation. (A) Effects of arcA, crp, and fnr mutations on H$_2$O$_2$ inhibition. Cultures of mid-log phase were properly diluted and plated onto LB plates. Paper discs of 6 mm in diameter loaded with 10 μl 5 M H$_2$O$_2$ were placed onto the bacterial lawn grown for 6 h. Results shown are from 18 h after the discs were in place. For data presentation, the relative sensitivity (RS) (y axis) of each mutant strain was calculated by normalizing its average diameter (n = 4) to the averaged diameter (n = 6) of the wild-type strain. Error bars represent SD. Asterisks indicate statistically significant difference (*P < 0.05; **P < 0.01; ***P < 0.001). (B) Survival assay. H$_2$O$_2$ was added to mid-log-phase cultures to the final concentration of 1 mM. After 5 min and 30 min, samples were properly diluted and plated onto LB plates. Colony counting was done after 24 h. The data reported represent the means (n = 3) ± SD.
In the sequences upstream of genes transcribed at levels significantly different between the wild type and ΔarcA strains (> 2-fold), ArcA-binding motifs were identified in general (Table S1). These data suggest that the observed differences at mRNA levels are most likely due to the arcA mutation, which overwhelms the impact of H₂O₂.

Most members of the OxyR regulon in the arcA mutant were still among the most substantially induced, including katB, katG-1, ahpC, ahpF, dps, SO1563 and SO3349 (glutathione peroxidase), ccpA, and ohr (Table S1) (16). Although SO1563 and ohr are under direct control of OhrR, they are also responsive to H₂O₂ (17). These data suggest that ArcA may not co-regulate regulons of OxyR and OhrR. Despite this, as the OxyR-mediated stress response is the most important mechanism for combating H₂O₂ in S. oneidensis, whether OxyR functions normally in the arcA-deficient background needs confirmation.

To this end, we compared expression of four genes (katB, ahpC, ahpF, katG-1) of the OxyR regulon between samples of the ΔarcA and ΔoxyR strains using a lacZ-reporter. While the ahpC and katG-1 genes are positively regulated by OxyR, the dps and katB genes are under its repression (16). As shown in Fig. 2B, expression levels of all these genes were concertedly upregulated in response to H₂O₂ in the oxyR+ background whereas none of them was responsive in the absence of OxyR. These data conclude that the OxyR regulon is independent of the Arc system in S. oneidensis.

ArcA has little influence on H₂O₂ degradation. In S. oneidensis, KatB is the catalase predominantly responsible for degradation of H₂O₂ (16). As a heme-containing protein, its activity is subjected to regulation of multiple aspects, such as iron homeostasis, heme biosynthesis, concentrations of endogenous inhibitors. To assess the H₂O₂ scavenging capability of the ΔarcA strain, we determined H₂O₂ consumption of this mutant. Cultures of the ΔarcA and its parental wild-type strains at mid-log phase were collected and sonicated. Aliquots of resulting extracts from cells of similar numbers were mixed with 0.5 mM H₂O₂ and concentrations of the remaining H₂O₂ were measured with time (Fig. 3A). As expected, the ΔkatB strain lost the ability to degrade H₂O₂ almost completely whereas the capacity of the ΔoxyR strain was enhanced significantly because of derepression of the katB gene (16). In the case of the ΔarcA strain, it had a H₂O₂-degrading dynamics indistinguishable from that of the wild-type. Moreover, the catalase activities in the wild-type and ΔarcA strains were compared. Proteins from cells immediately before and 30 min after the addition of 0.2 mM H₂O₂ were separated by native PAGE and stained for catalase activity. Again, the wild-type and ΔarcA strains exhibited abilities comparable to each other w/o

Figure 2. OxyR regulon is independent of the Arc system. (A) Clustered heat map of transcriptomes of the wild-type and ΔarcA mutant strains in response to H₂O₂. Cultures of mid-log phase were divided into two parts; one was collected as the untreated and the other was treated with 0.2 mM H₂O₂ and collected 5 min after as the treated. For each strain, the treated/untreated ratios were used for clustering and shown in color scale. Asterisk indicates genes that were significant different between two strains. (B) Expression of signature members of the OxyR regulon in the wild-type and ΔarcA mutant strains in response to H₂O₂. The data reported represent the means (n = 3) ± SD.
the H$_2$O$_2$ treatment whereas the oxyR mutant was not responsive to H$_2$O$_2$ (Fig. 3B). These data manifest that effects of the mutation in the arcA gene on the ability of S. oneidensis to scavenge H$_2$O$_2$ is negligible.

Loss of ArcA results in an increased take-up rate for H$_2$O$_2$. As shown above, neither function of OxyR nor H$_2$O$_2$ degradation ability in the ΔarcA strain is impaired, suggesting that the primary defense system for oxidative stress has no role in the elevated sensitivity to H$_2$O$_2$ caused by the arcA mutation. Given that there exists a H$_2$O$_2$ gradient across membranes and its diffusion rates have been found to be affected significantly by different membrane compositions (43–44), it is therefore possible that loss of ArcA improves diffusion of H$_2$O$_2$ across membranes. To test this, a fluorescent scopoletin-based assay was used to quantify H$_2$O$_2$. Exponentially growing cells of the wild-type and ΔarcA strains were treated with 0.5 mM H$_2$O$_2$ for 5 min and then filtered to separate cells from flow-through to obtain intracellular and extracellular fractions as reported before (42). After quantification of each fraction, the ratio between them was calculated. However, we found that intracellular H$_2$O$_2$ levels in both strains were too low to be determined confidently. To circumvent this difficulty, we removed the katB gene from the ΔarcA strain and compared to the ΔkatB single mutant. The double mutant strain had relatively stable H$_2$O$_2$ levels in the extracellular portion as the ΔkatB strain shown in Fig. 3A. This time, significant difference was observed. As shown in Fig. 4, extra/intra ratio of H$_2$O$_2$ in the arcA$^+$ strain (ΔkatB) was 2.8 times higher than in the arcA$^-$ strain (ΔarcAΔkatB). In addition, in the same experiment we found that the ΔoxyRΔkatB strain had a ratio similar to that of the ΔkatB strain, indicating that OxyR is dispensable in regulating H$_2$O$_2$ gradient across membranes. These results suggest that deletion of the arcA gene facilitates H$_2$O$_2$ uptake.

It has been reported that the most abundant porin OmpD is found to facilitate transport of H$_2$O$_2$ across the membrane in Salmonella enterica serovar Typhimurium (42). According to a previous report (45), S. oneidensis possesses an aquaporin and 6 general porins. To test the role of porins in the uptake of H$_2$O$_2$, we constructed a strain lacking all of these 7 porins and determined its H$_2$O$_2$ uptake capacity in the absence of the katB gene. As shown in Fig. 4, the extra/intra ratio of H$_2$O$_2$ of this mutant strain was comparable to that of the wild-type. These results, collectively, indicate that these porins are unlikely to have an important role in the uptake of H$_2$O$_2$ in S. oneidensis, and therefore not responsible for the hypersensitivity resulting from the ArcA loss.

Figure 3. The arcA mutation does not compromise the ability to decompose H$_2$O$_2$. (A) H$_2$O$_2$ consumption assay. H$_2$O$_2$ at 0.5 mM was added to mid-log-phase cultures, and the remaining H$_2$O$_2$ at the indicated time points was assayed. The data reported represent the means (n = 4) ± SD. (B) Catalase staining analysis. Cells were collected just before and 30 min after the addition of 0.2 mM H$_2$O$_2$. Proteins from the indicated cell lysates were separated by native PAGE and stained for catalase activity. Experiments were performed three times and similar results were obtained.
fluorescence assays in the failure of the leakage of the inner membrane) and PspA helps restore membrane integrity (47). To evaluate whether drastically diminished in the mutant. PspA is induced upon dissipation of the proton motive force (i.e.,
did not significantly impact the SDS and H2O2 sensitivities of either the wild-type or

Results showed, as presented in Fig. S1, that PspA produced at varying levels or the loss of the proton

Figure 4. In vivo diffusion of H2O2 into S. oneidensis. H2O2 levels were determined indirectly by specific fluorescence assays in the ΔkatB, ΔkatBΔarcA, ΔkatBΔoxyR, ΔkatBΔporin, and ΔkatB/SO1915. Δporin, ΔarcA/arcA, represent the strains lacking aquaporin as well as all 6 general porins and the genetically complemented strains of ΔarcA respectively. ΔkatB/SO1915 represents the ΔkatB strain carrying a copy of SO1915 under control of Ptac for overexpression with 0.5 mM IPTG. Exponentially growing cells were exposed to H2O2 (1 mM) for 5 min and peroxide levels were immediately determined in the extracellular (extra) and intracellular (intra) milieu and plotted as the extra/intra ratio. Asterisks indicate statistically significant difference (*P < 0.05; **P < 0.01; ***P < 0.001). Error bars indicate SD (n = 4).

Loss of ArcA results in a defect in the outer-membrane. One of possible mechanisms for increased H2O2 uptake rate is that the cell membranes are impaired. To test this possibility, we first performed sodium dodecyl sulfate (SDS) sensitivity assay. The result demonstrated that the arcA deletion drastically elevated susceptibility to SDS (Fig. 5A). In the presence of SDS at 0.1%, the sensitivity of the ΔarcA strain to the detergent evidently increased, and at 1% no growth was observed, contrasting a modest augment from the wild-type. Furthermore, the inhibitory effect of SDS on growth of liquid cultures was assessed (Fig. 5B). The loss of ArcA introduced a significant reduction in the aerobic growth rate in rich media, a scenario that is in excellent agreement with the results of previous studies (26,29,30).

Compared to those on plates, S. oneidensis cells in liquid media appeared much more sensitive to the detergent. No visible growth of the ΔarcA strain was observed when 0.1% was served for a 24-h incubation whereas growth of the wild-type was only modestly reduced. The inhibitory effect on the ΔarcA strain was evident even with 0.02% SDS. To further confirm that the inhibitory effect of SDS on growth is due to the cell envelope defect, we estimated the lysis of the wild-type and the ΔarcA cells by SDS. Cultures of mid-log phase (~0.4 of OD600) were treated with SDS of various concentrations and reduction of the optical density was monitored. When SDS was added to 1%, both the wild-type and the ΔarcA cultures were lysed completely (Fig. 5C). In contrast, significant differences in lysis levels were observed when lower concentrations were applied. SDS at 0.05% revealed a most dramatic distinction; the OD600 values of the ΔarcA culture reduced rapidly whereas the wild-type culture was barely affected.

Although enhanced sensitivity of Gram-negative bacterial cells to SDS is generally attributed to outer-membrane (OM) defects, further evidence is needed to prove whether OM as a permeability barrier is compromised. To confirm the defect in OM, we performed a lysozyme sensitivity assay. In Gram-negative bacteria, the structure of OM is stabilized by interactions between lipopolysaccharide and divalent metal cations; by chelating the latter, EDTA facilitates entry of lysozyme into the periplasm to digest peptidoglycan (46). As shown in Fig. 5D, significant time-dependent lysis of wild-type cells was observed only in the presence of both EDTA and lysozyme whereas EDTA alone was sufficient to induce severe disruption of the mutant cells, let alone rapid and dramatic lysis upon addition of both agents. This observation, in line with the enhanced sensitivity to SDS, indicates that the arcA mutation results in a defect in OM.

We then addressed whether the inner-membrane (IM) is defect or not. Microarray data given in Table S1 show a strong induction of the synthesis of PspA upon exposure of the wild-type to H2O2, which is drastically diminished in the mutant. PspA is induced upon dissipation of the proton motive force (i.e. leakage of the inner membrane) and PspA helps restore membrane integrity (47). To evaluate whether the failure of the arcA mutant to induce pspA expression is the reason for its increased sensitivity to H2O2, we assessed roles of PspA and/or the proton motive force in SDS and H2O2 resistance by using an IPTG-inducible expression vector pHGE-Ptac, which is routinely used in S. oneidensis (24,37,48–49).

Results showed, as presented in Fig. S1, that PspA produced at varying levels or the loss of the proton motive force did not significantly impact the SDS and H2O2 sensitivities of either the wild-type or arcA
mutant strains. We then repeated the sensitivity assay with Triton X-100, a nonionic surfactant that disrupts the inner membrane but not the outer membrane (50), and found that the wild-type and ∆arcA strains displayed comparable sensitivities (Fig. S1). These data together conclude that the arcA mutation does not interfere with the inner membrane.

Membrane-bound protease SO1915 in overabundance has a role in the cell envelope defect of the ∆arcA strain. The data presented thus far strongly suggest that the increased sensitivity of the ∆arcA strain to H2O2 is likely due to the impaired cell envelope. Given that the phenotype is clearly a result of the arcA mutation, we made an attempt to search for ArcA regulon members that may be involved in the cell envelope biosynthesis. Among genes possessing i) a predicted ArcA-binding motif within their upstream sequences and ii) a significant difference in expression (3-fold or above) between the wild-type and mutant strains (16,29), three serine protease genes, SO0867, SO1915, and aprE (SO3106) were particularly intriguing since they all displayed expression differences of over 30-fold (Table S1). Based on the protein subcellular localization prediction by using PSORTb, SO0867 and AprE are extracellular proteins but SO1915 likely resides in OM (data not shown).

Given that proteases are degradative enzymes which catalyze hydrolysis of target proteins (51), we reasoned that they may be detrimental when in overabundance. To estimate the impacts of these proteases on the oxidative stress response of S. oneidensis, we constructed mutants in which each of these genes was in-frame deleted. Neither growth under normal conditions (data not shown) nor susceptibility to H2O2 or SDS was affected significantly by the loss of each of these enzymes (Figs. 6A,6B). We then tested the ability of these proteins in overproduction to impact the cell envelope with r pHGE-Ptac. We fused the protease genes to Parc, resulting in pHGE-0867, pHGE-1915, and pHGE-3106. Each of these constructs was independently transferred by conjugation into the wild-type strain. In the presence of IPTG, cells carrying each of these plasmids were assayed for growth under normal conditions and susceptibility to H2O2 and SDS (Figs. 6A,6B). With IPTG at 0.5 mM (10–20-fold induction) (24,37,48–49),
no difference was observed from strains carrying pHGE-0867 or pHGE-3106. However, overexpression of the \( \text{SO1915} \) gene modestly altered the ability of the wild-type to cope with \( \text{H}_2\text{O}_2 \) and SDS although growth was not evidently affected. Interestingly, the \( \text{H}_2\text{O}_2 \) and SDS sensitivity of the \( \Delta \text{arcA} \) mutant carrying pHGE-1915 was not further increased (Fig. 6A). To confirm this, we examined the \( \text{H}_2\text{O}_2 \) uptake of the \( \Delta \text{katB} \) strain overexpressing the \( \text{SO1915} \) gene. Expectedly, the extra/intra ratio of \( \text{H}_2\text{O}_2 \) was decreased in the presence of 0.5 mM IPTG (Fig. 4). Furthermore, we deleted the \( \text{SO1915} \) gene from the \( \Delta \text{arcA} \) mutant and tested susceptibility of the resulting double mutant to \( \text{H}_2\text{O}_2 \) and SDS (Fig. 6B). With respect to \( \text{H}_2\text{O}_2 \) sensitivity the additional removal did not help significantly, but it did reduce the sensitivity to SDS to some extent, confirming that SO1915 in overabundance has a negative role in the cell envelope integrity. Despite this, it is evident that SO1915 is not the predominant factor accountable for the cell envelope defect resulting from the \( \text{arcA} \) deletion.

Although the presence of ArcA-binding motifs in front of all these three genes suggests that ArcA regulates their expression in a direct manner, a DNA-binding gel shift assay was performed for confirmation as performed before (29). As phosphorylation of ArcA is required for its specific binding, only ArcA (ArcA-P) phosphorylated by carbamoyl phosphate was used. The DNA fragments, approximately

Figure 6. Effects of production changes of three proteases of the ArcA regulon on the cell envelope defect. All assays were repeated at least three times and similar results were obtained. In A and B, strains without overexpressing proteases carried an empty vector. Overexpression was achieved by 0.5 mM IPTG. (A) Effects of expression changes of three proteases on \( \text{H}_2\text{O}_2 \) inhibition. Experiments were conducted and data were presented as described in Fig. 1. The values are the mean ± S.D. (error bars) (\( n = 4 \)). (B) Effects of expression changes of three proteases on the SDS resistance. (C) EMSA assay. Experiments were performed in the presence of 0, 1, or 2\( \mu \)M ArcA-P and 2–5 nM radiolabeled promoter DNA. 0.2\( \mu \)g/\( \mu \)l poly(dI·dC) was used in all these binding reactions to block non-specific interactions. Promoter region of \( \text{so0011} \) (\( \text{gyrB} \)) was included as negative control. The phosphorylation of the ArcA protein was done with carbamoyl phosphate.
300 bp in length centered by the predicted binding motif of the genes to be tested, were amplified with \(^{32}\)P end-labeled primers. It was found that phosphorylated ArcA protein significantly reduced the motility of fragments containing upstream sequences of SO0867, SO1915, and aprE but not the negative control fragment (Fig. 6C). In summary, SO1915 but not SO0867 or AprE in overabundance increases the sensitivity to H\(_2\)O\(_2\) and SDS although production of all these three proteases is under direct repression of ArcA.

**Cell envelope defect resulting from the arcA mutation appears unlikely to rely on a single gene.** Obviously, the defect in the cell envelope resulting from the arcA deletion is much more severe than that from the overproduction of SO1915 alone, suggesting that the role of ArcA in the cell envelope biosynthesis of *S. oneidensis* is profound and comprehensive. To identify other factors accountable for the cell envelope defect of the ΔarcA strain, we intended to introduce a new mutation, aiming at identification of suppressor genes. Plasmid pFAC, a mariner-based transposon vector widely used for construction of random insertion libraries in various bacteria, contains a modest promoter (P\(_{tn}\)) embedded in the transposable sequence (24,52). This additional feature, along with transposon, renders the vector suitability for screening for cryptic or quiescent operons in addition to knockout of active ones. The constructed library was spread on plates supplemented with the proper antibiotics and 0.4% SDS, which allows the wild-type to form colonies in 24 hours and prevents the ΔarcA strain from doing so completely. Unfortunately, from a total of ~300,000 individual insertion mutants, estimated by cfu on the SDS-free control plates, we did not obtain a single colony on screening plates. Additionally, we made an attempt with pHGT01, a derivative from pFAC with a robust promoter replacing P\(_{tn}\) (53). Still, no suppressor strain was obtained. Given that both vectors have been successfully utilized for this purpose by us and other research groups, this result implicates that the envelope defect resulting from the loss of ArcA unlikely relies on a single gene product.

**Discussion**

As ROS can be formed intracellularly when molecular oxygen interacts with redox enzymes (54), global regulators for respiration, especially the Arc system, have been implicated in bacterial oxidative stress response. In *E. coli*, *S. enterica* serovar Enteritidis, and *H. influenzae*, loss of the Arc system has been shown to result in elevated sensitivity to H\(_2\)O\(_2\) (9–10,42). However, the proposed mechanisms underlying the role played by Arc systems to combat the oxidative stress in these bacteria vary substantially. In *E. coli*, the Arc system is suggested to be important for the resistance to ROS through its pleiotropic effects such as those on metabolism, especially amino acid and/or protein assimilation and synthesis (10). On the contrary, specific effectors are accountable for the increased sensitivity to ROS in *S. enterica* serovar Typhimurium and *H. influenzae arcA* mutants (9,11,42). In the former, the most abundant porin, whose expression is negatively regulated by ArcA in the direct manner upon H\(_2\)O\(_2\) exposure, facilitates uptake of the oxidant. In the latter, the *dps* gene, encoding a well-characterized iron-storage protein of the OxyR regulon, is down-regulated in the absence of the arcA gene (1,9,16).

*S. oneidensis* is distinct from *E. coli* in global regulators that are utilized to control respiration. Although Crp is repeatedly shown to play a predominant role and Fnr appears negligible contrasting their *E. coli* counterparts (19–23), both of them have rather limited impacts on the response to oxidative stress. On the contrary, despite the substantial difference between regulons of *S. oneidensis* and *E. coli* Arc systems (29), we showed here that this is crucial for the bacterium to combat oxidative stress imposed by H\(_2\)O\(_2\). Like most, if not all, of bacteria, *S. oneidensis* is equipped a large number of the H\(_2\)O\(_2\) scavenging proteins, all of which are under the control of OxyR and OhrR (16–17). Concerted up-regulation of this entire scavenging repertoire upon H\(_2\)O\(_2\) exposure in the wild-type and *arcA* mutant strains implicates that the OxyR regulon is functionally independent of the Arc system. Given that the *dps* gene is the under the direct control of OxyR in *S. oneidensis* (16) and is not subjected to regulation by ArcA, it is therefore unlikely that the Arc system of *S. oneidensis* employs the same strategy as *H. influenzae* to confer the H\(_2\)O\(_2\) resistance (9). Additionally, the presented data manifest that porins in *S. oneidensis*, unlike in *S. enterica* serovar Typhimurium, form a major pathway for H\(_2\)O\(_2\) diffusion across OM (11).

Arc systems are found in \(\gamma\)-proteobacterial species and are now known to be involved in the diverse biological processes (55). Initially, the *arcA* gene of *E. coli* is recognized as the *dye* gene for mutation in this gene confers sensitivity to dyes such as toluidine blue O (TBO) and methylene blue (56). Both TBO and methylene blue are photosensitizers that facilitate ROS generation in the presence of light (57). It has been proposed that the Arc system limits the accumulation of oxygen radicals and the rate of utilization of endogenous reserves (58), which is applied to explain why the *E. coli* arcA mutant is sensitive to these redox dyes and reactive nitrogen species (RNS) such as nitric oxide (59,60). Although the *S. oneidensis bd* oxidase appears to function similarly in combating RNS, the *cydAB* operon is under the direct control of Crp rather than ArcA (24,61).

We showed that the cell envelope of *S. oneidensis* is impaired by the loss of the Arc system. The cell envelope defect, based on a substantially increased sensitivity to SDS, leads to an augment of the H\(_2\)O\(_2\) take-up rate. The enhanced sensitivity of the *S. oneidensis arcA* mutant to H\(_2\)O\(_2\)/SDS is at least in
part attributed to over-expressed SO1915, one of three serine proteases under the direct repression of ArcA. In contrast to the other two extracellular enzymes (SO0867 and AprE), SO1915 is located in OM. Conceivably, when overproduced it may exert a detrimental effect on the cell envelope by degrading proteins having a role in maintaining the envelope integrity. Efforts to test this notion by identifying its key targets are underway.

Under our test conditions, overproduction of SO1915 accounts for a small share of resistance of the arcA mutant to H$_2$O$_2$/SDS. We do not yet know the factors for the majority, even with transposon vectors capable of identifying genes of interest by either generating knockouts or overexpressing ones whose transcription is compromised in the absence of ArcA (24,51–52). Therefore, although the predicted S. oneidensis ArcA regulon includes a large number of genes encoding proteins in the functional category of cell envelope (20,29,62), our experience argues that none of these can be single-handedly responsible for the observed envelope defect. Nevertheless, we continue our effort to identify the important proteins for the defect as they are the key to better understand the underlying mechanism.

Predicted regulons of S. oneidensis (50 operons) and E. coli (82 operons) Arc systems differ from each other significantly, sharing only six operons (29,63). We previously proposed that in S. oneidensis ArcA-independent expression of conserved genes such as the tricarboxylic acid cycle (TCA) components may be largely due to the loss of ArcA-binding sites in their promoter regions. Similarly, as exemplified by the three proteases SO0867, SO1915, and AprE in this study, S. oneidensis ArcA may acquire control over new genes once an ArcA-binding site emerges. Moreover, the sensor protein ArcS (CaChe-PAS-PAS-HisKA) differs substantially from the E. coli ArcB (PAS-HisKA) in domain structure. In addition to one extra PAS domain (sensors of diverse signals), a unique CaChe domain may allow the ArcB (PAS-HisKA) in domain structure.

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In conclusion, the sensor protein ArcS (CaChe-PAS-PAS-HisKA) differs substantially from the E. coli ArcB (PAS-HisKA) in domain structure. In addition to one extra PAS domain (sensors of diverse signals), a unique CaChe domain may allow the protein to respond to signals other than redox changes (26–27,64). It is tempting to speculate, therefore, that the S. oneidensis Arc system senses different external stimuli than its E. coli counterpart.

References

1. Imlay, J.A. The molecular mechanisms and physiological consequences of oxidative stress: lessons from a model bacterium. Nat. Rev. Microbiol. 11, 443–454. (2013)
2. Mishra, S. & Imlay, J. Why do bacteria use so many enzymes to scavenge hydrogen peroxide? Arch. Biochem. Biophys. 525, 145–160. (2012)
3. Dubbs, J.M. & Mongkolsub, S. Peroxide-sensing transcriptional regulators in bacteria. J. Bacteriol. 194, 5495–5503. (2012)
4. Gu, M. & Imlay, J.A. The SoxRS response of Escherichia coli is directly activated by redox-cycling drugs rather than by superoxide. Mol. Microbiol. 79, 1136–1150. (2011)
5. Chiang, S.M. & Schellhorn, H.E. Regulators of oxidative stress response genes in Escherichia coli and their functional conservation in bacteria. Arch. Biochem. Biophys. 525, 161–169. (2012)
6. Lu, S., Killoran, P.B., Fang, F.C. & Riley, L.W. The global regulator ArcA controls resistance to reactive nitrogen and oxygen intermediates in Salmonella enterica Serovar Enteritidis. Infect. Immun. 70, 451–461. (2002)
7. Green, J. & Paget, M.S. Bacterial redox sensors. Nat. Rev. Microbiol. 2, 954–966. (2004)
8. Partridge, J.D., Poole, R.K. & Green, J. The Escherichia coli yhjA gene, encoding a predicted cytochrome c peroxidase, is regulated by FNR and OxyR. Microbiology 153, 1499–1509. (2007)
9. Wong, S.M.S., Alosgupalli, K.R., Ram, S. & Akerley, B.J. The ArcA regulon and oxidative stress resistance in Haemophilus influenzae. Mol. Microbiol. 67, 1375–1390. (2007)
10. Loui, C., Chang, A. & Lu, S. Role of the ArcAB two-component system in the resistance of Escherichia coli to reactive oxygen stress. BMC Microbiol. 9, 183. (2009)
11. Morales, E. et al. Hypochlorous acid and hydrogen peroxide-induced negative regulation of Salmonella enterica serovar Typhimurium ompW by the response regulator ArcA. BMC Microbiol. 12, 63. (2012)
12. Helmann, J. Prokaryotic redox switches. In Oxidative Stress and Redox Regulation. Jakob, U., and Reichmann, D. (eds): Springer, Netherlands, pp. 233–276. (2013)
13. Fredrickson, J.K. et al. Towards environmental systems biology of Shewanella. Nat. Rev. Microbiol. 6, 592–603. (2008)
14. Daly, M.I. et al. Accumulation of Mn(II) in Deinococcus radiodurans facilitates gamma-radiation resistance. Science 306, 1025–1028. (2004)
15. Yin, J. & Gao, H. Stress responses of Shewanella. Int. J. Microbiol. Article ID 863623, (2011)
16. Jiang, Y. et al. Protection from oxidative stress relies mainly on derepression of OxyR-dependent KatB and Dps in Shewanella oneidensis Isol. J. Bacteriol. 196, 445–458. (2014)
17. Li, N., Luo, Q., Jiang, Y., Wu, G. & Gao, H. Managing oxidative stresses in Shewanella oneidensis: intertwined roles of the OxyR and OhrR regulons. Environ. Microbiol. 16, 1821–1834. (2014)
18. Binnemkade, L., Teichmann, L. & Thormann, K.M. Iron triggers >So prophage induction and release of extracellular dna in Shewanella oneidensis MR-1 biofilms. Appl. Environ. Microbiol. 70, 5304–5316. (2014)
19. Maier, T.M. & Myers, C.R. Isolation and characterization of a Shewanella putrefaciens MR-1 electron transport regulator etraA mutant: Reassessment of the role of EtraA. J. Bacteriol. 183, 4918–4926. (2001)
20. Gao, H. et al. Physiological roles of ArcA, Crp, and Etra and their interactive control on aerobic and anaerobic respiration in Shewanella oneidensis. PLoS ONE 5, e13295. (2010)
21. Cruz-Garcia, C. et al. Fur (Etra) acts as a fine-tuning regulator of anaerobic metabolism in Shewanella oneidensis MR-1. BMC Microbiol. 11, 64. (2011)
22. Saffarini, D.A., Schultz, R. & Beliaev, A. Involvement of cyclic AMP (cAMP) and cAMP receptor protein in anaerobic respiration of Shewanella oneidensis. J. Bacteriol. 185, 3668–3671. (2003)
23. Dong, Y. et al. A Crp-dependent two-component system regulates nitrate and nitrite respiration in Shewanella oneidensis. PLoS ONE 7, e31643. (2012)
24. Fu, H. et al. Crp-dependent cytochrome bd oxidase confers nitrite resistance to Shewanella oneidensis. Environ. Microbiol. 15, 2198–2212. (2013)
25. Zhou, G. et al. Combined effect of loss of the caaO oxidase and Crp regulation drives Shewanella to thrive in redox-stratified environments. ISME J. 7, 1752–1763. (2013)
26. Lassak, J., Henche, A.-L., Binnemkade, L. & Thormann, K.M. ArcS, the cognate sensor kinase in an atypical Arc system of Shewanella oneidensis MR-1. Appl. Environ. Microbiol. 76, 3263–3274. (2010)
27. Shroff, N.P., Charnaria, M.A. & Saffarini, D.A. ArcB1, a homolog of Escherichia coli ArcB, regulates dimethyl sulfoxide reduction in Shewanella oneidensis MR-1. J. Bacteriol. 192, 1227–1230. (2010)
28. Gralnick, J.A., Brown, C.T. & Newman, D.K. Anaerobic regulation by an atypical Arc system in Shewanella oneidensis. Mol. Microbiol. 56, 1347–1357. (2005)
29. Gao, H., Wang, X., Yang, Z., Palzkill, T. & Zhou, J. Probing regulon of ArcA in Shewanella oneidensis MR-1 by integrated genomic analyses. BMC Genomics 9, 42. (2008)
30. Yuan, J., Wei, B., Lipton, M.S. & Gao, H. Impact of ArcA loss in Shewanella oneidensis revealed by comparative proteomics under aerobic and anaerobic conditions. Proteomics 12, 1957–1969. (2012)
31. Bertani, G. Lysogeny at mid-twentieth century: P1, P2, and other experimental systems. J. Bacteriol. 186, 595–600. (2004)
32. Jin, M. et al. Unique organizational and functional features of the cytochrome c maturation system in Shewanella oneidensis. PLoS ONE 8, e75610. (2013)
33. Wu, L., Wang, J., Tang, P., Chen, H. & Gao, H. Genetic and molecular characterization of flagellar assembly in Shewanella oneidensis. PLoS ONE 6, e21479. (2011)
34. Wolff, S.P. Ferrous ion oxidation in presence of ferric indicator xylenol orange for measurement of hydroperoxides. Methods Enzymol. 233, 182–189. (1994)
35. Gao, H. et al. Global transcriptome analysis of the heat shock response of Shewanella oneidensis. J. Bacteriol. 186, 7796–7803. (2004)
36. Gao, H. et al. Generation and validation of a Shewanella oneidensis MR-1 clone set for protein expression and phage display. J. Bacteriol. 183, 22983. (2008)
37. Fu, H., Jin, M., Ju, L., Mao, Y. & Gao, H. Evidence for function overlapping of CymA and the cytochrome bc complex in the Shewanella oneidensis nitrate and nitrite respiration. Environ. Microbiol. DOI: 10.1111/1462-2920.12457. (2014)
38. Clare, D.A., Duong, M.N., Darr, D., Archibald, F. & Fridovich, I. Effects of molecular oxygen on detection of superoxide radical with nitroblue tetrazolium and on activity stains for catalase. Anal. Biochem. 140, 532–537. (1984)
39. Boveris, A., Martino, E. & Stoppani, A.O.M. Evaluation of the horseradish peroxidase-scopoletin method for the measurement of hydrogen peroxide formation in biological systems. Anal. Biochem. 80, 155–158. (1977)
40. Yu, N.Y. et al. PSORTb 3.0: improved protein subcellular localization prediction with refined localization subcategories and predictive capabilities for all prokaryotes. Bioinformatics 26, 1608–1615. (2010)
41. Thomas-Chollier, M. et al. RSAT 2011: regulatory sequence analysis tools. Nucleic Acids Res. 39, W86–W91. (2011)
42. Calderón, I.L. et al. Response regulator ArcA of Salmonella enterica serovar Typhimurium downregulates expression of OmpD, a porin facilitating uptake of hydrogen peroxide. Res. Microbiol. 162, 214–222. (2011)
43. Seaver, L.C. & Imlay, J.A. Hydrogen peroxide fluxes and compartmentalization inside growing Escherichia coli. J. Bacteriol. 183, 7182–7189. (2001)
44. Bierner, G.P. & Chaumont, F. Aquaporin-facilitated transmembrane diffusion of hydrogen peroxide. Biochim. Biophys. Acta. 1840, 1596–1604. (2014)
45. Yuan, J., Wei, B., Shi, M. & Gao, H. Functional assessment of EnvZ/OmpR two-component system in Shewanella oneidensis. PLoS ONE 6, e23701. (2011).
46. Stanley, N. R., Findlay, K., Berks, B. C. & Palmer, T. Escherichia coli strains blocked in tat-dependent protein export exhibit pleiotropic defects in the cell envelope. J. Bacteriol. 183, 139–144. (2001)
47. Kleerebezem, M., Criezelaard, W. & Tommassen, J. Involvement of stress protein PspA (phage shock protein A) of Escherichia coli in maintenance of the protonmotive force under stress conditions. EMBO J. 15, 162–171. (1996)
48. Luo, Q., Dong, Y., Chen, H. & Gao, H. Mislocalization of rksA protein peta predominantly accounts for the aerobic growth defect of tat mutants in Shewanella oneidensis. PLoS ONE 8, e62064. (2013)
49. Shi, M., Gao, T., Ju, L., Yao, Y. & Gao, H. Effects of FhrBC on flagellar biosynthesis of Shewanella oneidensis. Mol. Microbiol. 93, 1269–1283. (2014)
50. Seddon, A. M., Curnow, P. & Booth, P. J. Membrane proteins, lipids and detergents: not just a soap opera. Biochim. Biophys. Acta. 1666, 105–117. (2004.)
51. Page, M., & Di Cera, E. Serine peptidases: Classification, structure and function. Cell. Mol. Life Sci. 65, 1220–1236. (2008)
52. Wong, S.M. & Mekalanos, J.J. Serine peptidase footprinting with mariner-based transposition in Pseudomonas aeruginosa. Proc. Natl. Acad. Sci. USA 97, 10191–10196. (2000)
53. Yin, J. et al. Regulation of nitrate resistance of the cytochrome cbb oxidase by cytochrome c ScyA in Shewanella oneidensis. Microbiology Open, doi: 10.1002/mbo3.224. (2014)
54. Korshunov, S. & Inlay, J.A. Two sources of endogenous hydrogen peroxide in Escherichia coli. Mol. Microbiol. 75, 1389–1401. (2010)
55. Dong, Y., Wan, F., Yin, J. & Gao, H. Ecological roles of Arc signal transduction system revealed by evolutionary genetics analysis. J. Bacteriol. Mycol. 1(2), 7. (2014)
56. Iuchi, S. & Lin, E.C. arcA (dye), a global regulatory gene in Escherichia coli mediating repression of enzymes in aerobic pathways. Proc. Natl. Acad. Sci. USA 85, 1888–1892. (1988)
57. Ruiz, J.A., Fernández, R.O., Nikel, P.L., Méndez, B.S. & Pettinari, M.I. dye (arc) mutants: insights into an unexplained phenotype and its suppression by the synthesis of poly (3-hydroxybutyrate) in Escherichia coli recombinants. FEBS Microbiol. Lett. 258, 55–60. (2006)
58. Park, S., Chao, G. & Günsalus, R. Aerobic regulation of the sacABCD genes of Escherichia coli, which encode alpha-ketoglutarate dehydrogenase and succinyl coenzyme A synthetase: roles of ArcA, Fnr, and the upstream sdhCDAB promoter. J. Bacteriol. 179, 4138–4142. (1997)
59. Alvarez, A.F., Malpica, R., Contreras, M., Escamilla, E. & Georganellis, D. Cytochrome d but not cytochrome d descreses the toluidine blue growth sensitivity of arc mutants of Escherichia coli. J. Bacteriol. 192, 391–399. (2010)
60. Mason, M.G. et al. Cytochrome bd confers nitric oxide resistance to Escherichia coli. Nature Chem. Biol. 5, 94–96. (2009)
61. Zhang, H. et al. Impacts of nitrate and nitrogen on physiology of Shewanella oneidensis. PLoS ONE 8, e62269. (2013)
62. Wang, X. et al. A high-throughput percentage-of-binding strategy to measure binding energies in DNA–protein interactions: application to genome-scale site discovery. Nucleic Acids Res. 36, 4863–4871. (2008)
63. Liu, X. & De Wulf, P. Probing the ArcA-P modulon of Escherichia coli by whole genome transcriptional analysis and sequence recognition profiling. J. Biol. Chem. 279, 12588–12597. (2004)
64. Taylor, B.L. & Zhulin, I.B. PAS domains: internal sensors of oxygen, redox potential, and light. Microbiol. Mol. Biol. Rev. 63, 479–506. (1999)

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Author Contributions
H.G. conceived the idea and designed the project. F.W., Y.M., Y.D., L.J. and G.W. carried out the experiments. F.W., G.W. and H.G. analyzed data. F.W., G.W. and H.G. wrote the paper.

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