Physiological Roles of ArcA, Crp, and EtrA and Their Interactive Control on Aerobic and Anaerobic Respiration in *Shewanella oneidensis*

Haichun Gao1,2*, Xiaohu Wang3*, Zamin K. Yang4, Jingrong Chen2, Yili Liang2, Haijiang Chen1, Timothy Palzkill3, Jizhong Zhou2*

1 Institute of Microbiology and College of Life Sciences, Zhejiang University, Hangzhou, Zhejiang, China, 2 Institute for Environmental Genomics and Department of Botany and Microbiology, University of Oklahoma, Norman, Oklahoma, United States of America, 3 Department of Pharmacology & Department of Molecular Virology and Microbiology, Baylor College of Medicine, Houston, Texas, United States of America, 4 Environmental Sciences Division, Oak Ridge National Laboratory, Oak Ridge, Tennessee, United States of America

Abstract

In the genome of *Shewanella oneidensis*, genes encoding the global regulators ArcA, Crp, and EtrA have been identified. All these proteins deviate from their counterparts in *E. coli* significantly in terms of functionality and regulon. It is worth investigating the involvement and relationship of these global regulators in aerobic and anaerobic respiration in *S. oneidensis*. In this study, the impact of the transcriptional factors ArcA, Crp, and EtrA on aerobic and anaerobic respiration in *S. oneidensis* were assessed. While all these proteins appeared to be functional in vivo, the importance of individual proteins in these two major biological processes differed. The ArcA transcriptional factor was critical in aerobic respiration while the Crp protein was indispensable in anaerobic respiration. Using a newly developed reporter system, it was found that expression of arcA and etrA was not influenced by growth conditions but transcription of crp was induced by removal of oxygen. An analysis of the impact of each protein on transcription of the others revealed that Crp expression was independent of the other factors whereas ArcA repressed both etrA and its own transcription while EtrA also repressed arcA transcription. Transcriptional levels of arcA in the wild type, crp, and etrA strains under either aerobic or anaerobic conditions were further validated by quantitative immunoblotting with a polyclonal antibody against ArcA. This extensive survey demonstrated that all these three global regulators are functional in *S. oneidensis*. In addition, the reporter system constructed in this study will facilitate in vivo transcriptional analysis of targeted promoters.

Introduction

Depending on the availability of electron donors and acceptors, facultative anaerobes such as *Escherichia coli* adopt three different metabolic modes: aerobic respiration, anaerobic respiration, and fermentation [1–2]. Aerobic respiration with oxygen as the terminal electron acceptor (EA) is the most productive mode due to complete oxidation of a growth substrate while anaerobic respiration can only oxidize a substrate partially with alternative electron acceptors, such as nitrate. Fermentation is the least productive process because only substrate level phosphorylation can occur. As a result, aerobic respiration is preferred over anaerobic respiration, which in turn is preferred over fermentation [3]. Since molecular oxygen confers enormous energetic benefits, it is not surprising that changes in its availability lead to substantial changes in *E. coli* physiology. Several sensing systems are employed in *E. coli* to monitor environmental oxygen and cellular redox state. The switch between aerobic and anaerobic metabolism is controlled primarily by the Fnr (fumarate nitrate regulator) transcription factor and the Arc (aerobic respiration control) two-component regulatory system [3]. Fnr, synthesized under both aerobic and anaerobic conditions, is able to sense oxygen directly using its [4Fe-4S]2+ cluster whereas the Arc system senses oxygen indirectly [4]. After sensing changes in the redox state of the quinone pool elicited by oxygen limitation, ArcB, the sensor kinase of the system, autophosphorylates and then transphosphorylates the response regulator ArcA [5–6].

*Shewanella oneidensis* MR-1 is a facultative Gram-negative anaerobe with remarkable anaerobic respiration abilities that allow the use of a diverse array of terminal EAs [7]. Although little is known about how this bacterium adopts different metabolic modes in response to the availability of oxygen, surprising observations have been made. First, *S. oneidensis* does not ferment, although genes encoding many enzymes of mixed acid fermentation are present in the genome [8]. Second, EtrA (electron transport regulator A), the analog of *E. coli* Fnr, appears to have no
significant role in mediating gene expression in response to oxygen availability [9–10]. Third, it is evident that Crp (cyclic-AMP receptor protein) is crucial in anaerobic respiration because crp mutants are defective in utilizing several EAs, including FeO(OH), MnO2, nitrate, fumarate, and dimethyl sulfoxide (DMSO) [11].

Fourth, _S. oneidensis_ possesses an atypical Arc system in which function of ArcB is fulfilled by two proteins ArcS and HpA [12]. Unlike the Arc system in _E. coli_, this atypical system appears to be important in aerobic respiration and is not involved in regulation of TCA genes [13].

In the present study, we report results from experiments designed to reveal the involvement of three global regulators (ArcA, Crp, and EtrA) in aerobic and anaerobic respiration in _S. oneidensis_. For this purpose, seven strains, each of which has at least one of these three genes deleted, were constructed and characterized. In addition, a LacZ reporter system was developed for Gram-negative bacteria lacking a lacZ analog to investigate the interactive control among these regulators at the transcriptional level. The results were validated by quantitative western blotting with antibodies against _S. oneidensis_ ArcA proteins. The results indicate that these transcription factor proteins are functional in vivo and respond with unique characteristics to the availability of oxygen and other transcription proteins.

**Results**

**Generation of arcA, crp, and etrA single, double, and triple deletion mutants**

Individual chromosomal mutants of _arcA, crp_, and _etrA_ have been generated and characterized [9,11,13–14]. While an _arcA_ mutant, designated as JZ3988K (ArcA), was constructed and validated as a deletion, the _crp_ and _etrA_ constructs reported previously were insertion mutants. Unfortunately, the first _etrA_ mutant was later invalidated [9,14]. As a result, concerns about insensational mutagenesis in _S. oneidensis_ have been raised, especially with MR-1R as the parental strain. MR-1R carries a mutation resulting in a lacZ allele which causes a decreased menaquinone pool [15]. This complicates physiological observations on growth under anaerobic conditions because the menaquinone pool is the electron source for terminal reductases in anaerobic respiration.

Given their importance in respiration of _E. coli_ and _S. oneidensis_, the creation and validation of a collection of mutants in which one or more of the _arcA, crp_, and _etrA_ genes is (are) deleted would be indispensable for a quantitative characterization of global transcriptional regulation. To this end, the _crp_ and _etrA_ single deletion mutants were constructed, named JZ0624 (ArcA) and JZ2356 (ArcAcrp) respectively. With one of these single mutants as the parental strain, double mutants JZ3988K-0624 (ArcAcrp), JZ3988K-2356 (ArcAcrpETR), and JZ0624-2356 (arcAcrp) were generated by simply repeating the mutagenesis procedure. The deletion(s) in all of these strains as listed in Table 1 were verified by PCR and DNA sequencing.

**Heme c levels in mutants**

It has been reported that _S. oneidensis_ contains 42 genes for predicted _c_-type cytochromes [19] and the latest analysis suggests that the genome possesses 41 genes encoding intact _c_-type cytochrome proteins [20]. As the main components of respiratory electron transport chains, it is conceivable that cellular levels of heme _c_ (components of _c_-type cytochromes) could be affected by mutations in the genes encoding the major transcriptional regulatory factors controlling bacterial aerobic and anaerobic respiration. To assess impacts of ArcA, EtrA and Crp on cellular levels of _c_-type cytochromes, the wild-type and mutant cells grown to an OD<sub>600</sub> of 0.6 under aerobic conditions and to an OD<sub>600</sub> of 0.25 under anaerobic conditions were collected and cellular levels of heme _c_ in these samples were examined and results were shown in Fig. 1A. The wild type cells grown under aerobic and anaerobic conditions contained approximately 1.8 µM and 2.4 µM concentration of heme _c_ per g of protein, respectively. Levels of cellular heme _c_ in mutant strains varied significantly, with an average of 0.9/1.05 (aerobic/anaerobic, the same below) in any mutant devoid of _crp_, an average of 1.5/1.8 in _ArcA_ and _ArcAcrpETR_, and an average of 1.8/2.4 in _ArcAcrpETR_. To facilitate the comparison of mutation effects on the cellular heme _c_ levels, relative heme _c_ levels of each in mutant were presented as the ratios of the absolute amount of heme _c_ of each mutant vs. MR-1 under the same growth conditions (Fig. 1B). All of these results demonstrated that 1) the amount of heme _c_ in each strain was higher when grown under anaerobic conditions; 2) relative heme _c_ level in each mutant appeared to be similar irrespective of growth conditions although phenotypes of certain mutants were only observed under either aerobic or anaerobic condition; 3) mutants in which _crp_ was deleted exhibited significantly lower levels of heme _c_ than the wild type, whereas mutations in _arcA_ showed some mild negative influence on heme _c_ synthesis and _etra_ did not affect heme _c_ levels.

**Physiological analysis of the mutants**

The single, double and triple deletion mutant strains were subjected to physiological characterization as performed previously for the _arcA_ deletion mutant JZ3988K [13]. The respiratory conditions tested in the characterization included: (i) aerobiciosis, and (ii) anaerobiosis with a variety of electron acceptors (EA) including fumarate (20 mM), nitrate (3 mM), dimethyl sulfoxide (DMSO 20 mM), trimethylamine _N_-oxide (TMAO 20 mM), thiosulfate (3 mM), MnO2 (5 mM), ferric citrate (10 mM), and FeO(OH) (10 mM).

In order to precisely assign observed phenotypes to individual mutations, physiological differences of three single mutants under aerobic and anaerobic conditions were examined. The results presented in Fig. 2 indicate that a mutation in _arcA_ results in a substantially slower growth rate of cells under aerobic conditions while the growth rate of strains carrying mutation in either _etra_ or _etra_ was identical to that of wild type MR-1, consistent with previous reports [1,11,13–14]. Dissolved oxygen in all cultures was monitored as done previously with the _ArcA_ strain and the same result was obtained (data not shown) [13]. In all cases, DO decreased quickly at the early stage, reached the lowest point at the mid-log phase and remained at the level until the late stationary phase, suggesting that DO is a function of the cell density but not related to the genotype of individual strains. Not surprisingly, the double mutants (ArcAcrp and ArcAcrpETR) and the triple mutant (ArcAcrpETRhetra) shared a similar phenotype of slower aerobic growth with the _ArcA_ strain while the double mutant ArcAcrpETR was not defective in growth rate (data not shown). These results strongly suggest that _arcA_ but not _crp_ or _etra_ plays a substantial role in the bacterial aerobicism.

Under anaerobic growth conditions, the utilization of EAs by the mutants was investigated by following methods. The ability to utilize fumarate, nitrate, DMSO, TMAO, or thiosulfate was assayed by measuring culture turbidity. Utilization of MnO2, ferric citrate, and cobalt(III)-EDTA was assayed by the color change of cultures and validated by chemical analysis as described in Methods. The results clearly revealed that Csp plays an important role in _S. oneidensis_ anaerobic respiration (Table 2). Among the tested EAs, only TMAO allows cells of the _Arc_ strain.
Table 1. Strains and plasmids used in this study.

| Strain or plasmid | Description | Reference or source |
|-------------------|-------------|---------------------|
| **E. coli strain** |             |                     |
| BL21             | F' ompT hsdS2(rK– mcrA) gal dcm (DE3) | GE Healthcare |
| WM3064           | Donor strain for conjugation; ΔdopA | [16] |
| **S. oneidensis strains** |             |                     |
| MR-1             | Wild-type | ATCC 700550 |
| JZ3988K          | arcA deletion mutant derived from MR-1; ΔarcA | [13] |
| JZ0624           | crp deletion mutant derived from MR-1; Δcrp | This study |
| JZ2356           | etrA deletion mutant derived from MR-1; ΔetrA | This study |
| JZ3988-0624      | arcA and crp double deletion mutant derived from MR-1; ΔarcAΔcrp | This study |
| JZ3988-2356      | arcA and etrA double deletion mutant derived from MR-1; ΔarcAΔetrA | This study |
| JZ0624-2356      | crp and etrA double deletion mutant derived from MR-1; ΔcrpΔetrA | This study |
| JZ3988-0624-2356 | arcA, crp, and etrA triple deletion mutant derived from MR-1; ΔarcAΔcrpΔetrA | This study |
| **Plasmids**     |             |                     |
| pD53.0           | Ap’, Gm’, derivative from suicide vector pCVD442 | [17] |
| pD5-ARCAK        | pD53.1 containing the PCR fragment for deleting arcA | [13] |
| pD5-CRP          | pD53.1 containing the PCR fragment for deleting crp | This study |
| pD5-ETRA         | pD53.1 containing the PCR fragment for deleting etrA | This study |
| pCM62            | Base plasmid for constructing a reporter system | [18] |
| pBlueStar-1      | Full length of lacZ gene template | Novagen |
| pTP327           | pCM62 with a full length lacZ gene | This study |
| pTP327           | pTP327 without Plac promoter | This study |
| pTP327-ARCAp     | pTP327 containing the S. oneidensis arcA promoter | This study |
| pTP327-CRPa      | pTP327 containing the S. oneidensis crp promoter | This study |
| pTP327-ETRAP     | pTP327 containing the S. oneidensis etrA promoter | This study |

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to grow, as reported previously (Table 2) [11]. In contrast, the ΔetrA strain did not show distinguishable defects in growth with any of the tested EAs compared to the parental strain MR-1 (Table 2). It was reported previously that an insertional etrA mutant differed noticeably in utilization of fumarate and nitrate from MR-1 [14]. For verification, an IC (Ion Chromatography) analysis on nitrate and nitrite levels in cultures of the single mutant and the double mutant. These findings suggest that ArcA plays a more important role in carbon metabolism than Crp and EtrA while the role of EtrA appears negligible.

Phenotypic microarray analysis of the mutants

The canonical Crc as in E. coli is the primary sensor and transcriptional regulator of carbon metabolism [22]. Although function shift of S. oneidensis ArcA, Crc, and EtrA appears to be substantial, an examination of their canonical roles is much needed. To this end, the ability of the wild-type and all mutant strains to metabolize 190 different carbon sources under aerobic conditions was tested using PM1 phenotype microarrays from Biolog (www.biolog.com/pdf/PM1-PM10.pdf). Wild type MR-1 displayed positive reaction with 15 carbon sources presented in Table 3. While both the ΔarcA and ΔcrpΔarcA strains were able to utilize all these 15 carbon sources, strains carrying a deletion in arcA were metabolically more restricted. It is also evident that the ΔarcA single mutant and the ΔarcAΔectA double mutant were virtually the same in their ability to metabolize these carbon sources. The Δcrp strain, however, appeared to be able to rescue defects of the ΔarcA strain on some carbon sources, such as glycyrl-L-glutamic acid, tween 40, and tween 80 as revealed by the ΔarcAΔcrp and triple mutants. These findings suggest that ArcA plays a more important role in carbon metabolism than Crc and EtrA while the role of EtrA appears negligible.

Development and validation of a lacZ reporter system in S. oneidensis

In E. coli, ArcA, Crc, and Fnr[EtrA] are important components of transcriptional regulatory networks and inter-regulation mechanisms among the transcription factors have been reported [23]. To explore whether such mechanisms are present in S. oneidensis, a β-galactosidase reporter assay was established for studying in vivo transcriptional regulation in S. oneidensis. A plasmid, pCM62, suitable for developing an E. coli LacZ reporter system has been widely used in a variety of Gram-negative bacteria including S. oneidensis [18,24]. While the plasmid has been exploited for many different purposes, it cannot be directly applied to S. oneidensis as an E. coli LacZ reporter system for following two reasons. First, the S. oneidensis genome lacks a lacZ homolog gene, thus it is not able to
provide lacZβγδ to complement the lacZ expression from an inserted S. oneidensis promoter subjected to examination. It may be particularly important for this study because the Plac promoter has been shown to be regulated by Crp in E. coli [25].

To develop an E. coli LacZ reporter system for S. oneidensis, the lacZ gene of pCM62 was replaced with a full length E. coli lacZ gene generated by PCR with the template and primers listed in Table S1 in Supporting Information (Fig. 3). The resulting plasmid, pTP325, was further modified by substituting the Plac promoter located in front of the full length E. coli lacZ gene with a synthetic oligo (listed in Table S1), resulting in the promoterless pTP327. To test utility of pTP327 as a promoter-probe vector, the arcA, crp, or etrA promoters of S. oneidensis were transcriptionally fused to lacZ within pTP327, resulting in pTP327-ArcA, pTP327-Crp, and pTP327-EtrA, respectively. These plasmids, along with pCM62, pTP325, and pTP327, were individually introduced into S. oneidensis MR-1 for validation. The β-galactosidase activities in cells grown under aerobic conditions were measured as presented in Table 4. While similar reporter activities were obtained with pCM62 and pTP325 in E. coli under the same conditions (presence/absence of 0.5 mM IPTG), reporter activities of these plasmids in S. oneidensis differed significantly. Expectedly, pCM62 failed to produce β-galactosidase activity due to the lack of full-sized LacZ and pTP325 expressed lacZ constitutively because of the lack of LacI in the bacterium. This confers an advantage of blue-white screening to pTP325 over pCM62 as an expression vector in S. oneidensis and other Gram-negative bacteria without a lacZ homolog in their genome. A higher background β-galactosidase activity from pTP327 in E. coli than S. oneidensis was likely due to the fact that E. coli hosted a higher copy number of the plasmid because of its ColE1 ori. Other origins, which allow S. oneidensis to maintain the plasmid, restricted a high copy number in the microorganism [18].

Expression of lacZ from inserted promoters within pTP327-ArcA, pTP327-Crp, and pTP327-EtrA was at least 10 times above the background. The extent of elevation appeared to be promoter specific. These results suggest that pTP327 is able to fulfill the need for specific mini-promoter systems in S. oneidensis. Note that this promoterless system should function not only in S. oneidensis but also any Gram-negative bacteria compatible with the broad-host ori within the plasmid.

**In vivo inter-regulation of ArcA, Crp, and EtrA**

The in vivo inter-regulation of arcA, crp and etrA gene transcription by ArcA, Crp, and EtrA was determined by comparing the expression pattern of the arcA, crp, or etrA-lacZ reporter constructs in S. oneidensis MR-1 with that in the mutant strains under aerobic, or anaerobic TMAO growth conditions. Since the background was far below the β-galactosidase activity obtained from plasmids containing inserted promoters, it was omitted in data analysis. Both the absolute β-galactosidase promoter activity (APA) of each promoter construct (Fig. 4A, 4C) and relative promoter activity (RPA) (Fig. 4B, 4D), which was calculated by normalizing APA of each mutant to APA of the wild type under the same condition were shown.
The deletion of either ArcA or EtrA but not in anaerobic TMAO growth [13]. However, ArcA has been shown to be deeply involved in bacterial conditions (Fig. 4A, 4C). This is somewhat unexpected because wild type background was not strongly affected by growth proteins function as repressors. In contrast, the absence of the ArcA or EtrA gene products, suggesting that both arcA or etrA resulted in a significant impact on the arcA promoter activity (Fig. 4B, 4D). The relative activities of the arcA promoter were elevated more than 10 times in the arcA crp and arcA etrA double or the triple mutant. These results may indicate an epistasis relationship between ArcA and EtrA.

The reporter results with the crp promoter are completely different from those observed with the arcA promoter. In this case, the transcription activity of the crp promoter was hardly affected by any deletion mutation (Fig. 4B, 4D). However, the presence of oxygen in the culture resulted in an approximately 2.5-fold decrease in the crp promoter activity, consistent with the idea that the Crp protein has a larger role in anaerobiosis than aerobiosis in S. oneidensis [11]. Based on the observation that no change in growth phenotype resulted from an etrA mutation and EtrA was present in the wild-type under aerobic and anaerobic conditions as revealed by proteomics (unpublished results), thus a constant activity from the etrA promoter was expected under tested conditions. Indeed, transcriptional activity of the etrA promoter was not sensitive to growth conditions. Surprisingly, however, the arcA deletion strains exhibited a substantial increase in etrA promoter activity, suggesting ArcA represses etrA transcript. On the contrary, etrA transcription was not affected by the etrA or crp deletions under the conditions tested (Fig. 4B, 4D).

### Table 2. Growth differences of mutants compared to the wild type under anaerobic conditions.

| Strain       | Electron Acceptor (EA) | Fumarate | DMSO | TMAO | Nitrate | FeO(OH) | Thiosulfate | Fe-Citrate | MnO₂ |
|--------------|------------------------|----------|------|------|---------|---------|-------------|------------|------|
| ΔarcA        | N                      | D        | N    | N    | N       | N       | N           | N          | N    |
| Δcrp         | D                      | D        | N    | D    | D       | D       | D           | D          | D    |
| ΔetrA        | N                      | N        | N    | N    | N       | N       | N           | N          | N    |
| ΔarcAΔcrp    | D                      | N        | D    | D    | D       | D       | D           | D          | D    |
| ΔarcAΔetrA   | N                      | D        | N    | N    | N       | N       | N           | N          | N    |
| ΔcrpΔetrA    | D                      | D        | N    | N    | D       | D       | D           | D          | D    |
| Triple       | D                      | D        | N    | D    | D       | D       | D           | D          | D    |

*Used in defined medium supplemented with 20 mM lactate, concentrations of EAs refer to the text.

*Growth on or reduction of the electron acceptor used is defective (D).

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### Table 3. Carbon utilization of MR-1 and mutation strains by phenotypic microarray analysis.

| Metabolite               | ΔarcA | Δcrp | ΔetrA | ΔarcAΔcrp | ΔarcAΔetrA | ΔcrpΔetrA | Triple |
|--------------------------|-------|------|-------|------------|------------|-----------|--------|
| Glycyl-L-Aspartic Acid   | N     | 0.85±0.13 | 1.13±0.17 | N          | N          | 2.63±0.27 | N      |
| Glycyl-L-glutamic Acid   | N     | 1.93±0.20 | 1.26±0.09 | 0.94±0.13 | N          | 2.12±0.31 | 1.29±0.08 |
| N-Acetyl-D-Glucosamine   | 0.70±0.09 | 2.74±0.27 | 1.71±0.21 | 1.78±0.17 | 0.58±0.07 | 2.24±0.33 | 2.27±0.29 |
| Tween 20                 | 0.49±0.07 | 1.55±0.12 | 0.94±0.08 | 0.54±0.04 | 0.60±0.03 | 1.18±0.12 | 0.72±0.12 |
| Tween 40                 | N     | 2.16±0.11 | 1.28±0.08 | 0.71±0.07 | N          | 1.47±0.14 | 0.70±0.09 |
| Tween 80                 | N     | 1.92±0.21 | 1.13±0.11 | 0.76±0.08 | N          | 1.93±0.17 | 1.00±0.07 |
| α-Keto-Butyric Acid      | N     | 1.89±0.31 | 2.36±0.34 | 1.02±0.15 | N          | 2.33±0.16 | N      |
| Pyruvic Acid             | N     | N     | 1.10±0.12 | N          | N          | 1.13±0.15 | N      |
| L-Lactic Acid            | 0.50±0.07 | 1.84±0.19 | 1.98±0.12 | N          | 0.46±0.04 | 1.36±0.10 | N      |
| Methyl Pyruvate          | 0.92±0.06 | 2.13±0.04 | 2.16±0.06 | 0.64±0.07 | 1.06±0.10 | 1.44±0.17 | 1.03±0.06 |
| 2-Deoxy Adenosine        | N     | N     | 1.90±0.01 | N          | N          | 1.44±0.09 | N      |
| Uridine                  | 0.81±0.11 | 1.41±0.15 | 2.55±0.09 | 0.99±0.08 | 1.02±0.09 | 2.47±0.35 | 1.83±0.10 |
| Adenosine                | 0.81±0.05 | 1.79±0.07 | 1.92±0.11 | 1.24±0.09 | 1.09±0.06 | 1.58±0.16 | 1.58±0.13 |
| Inosine                  | 1.06±0.07 | 2.06±0.30 | 1.99±0.21 | 1.42±0.10 | 1.58±0.16 | 1.69±0.11 | 1.70±0.16 |
| Gelatin                  | N     | 2.18±0.12 | 1.99±0.17 | N          | N          | 1.64±0.18 | N      |

*Relative values were presented as signal reading of each mutant/signal reading of the wild-type strain. N represents negative results from signal reading of certain mutant with specific metabolite.

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It is apparent that the β-galactosidase reporter experiments for these promoters revealed some interesting findings. The crp gene is the most transcriptionally active with β-galactosidase levels at least two times higher than those of arcA and etrA (Fig. 4A, 4C). It is also the only gene whose expression is significantly influenced by oxygen but not by deletions of the major global regulator proteins, including Crp itself. Although the etrA gene was transcribed at the lowest level, its product appeared to be functional as evidenced by its role in the repression on the arcA promoter. Both arcA and etrA promoters were repressed by ArcA, but availability of oxygen hardly influenced transcription of these two genes.

**In silico** analysis on interaction between ArcA, Crp, and EtrA and their promoters

The experimental data presented thus far have established the interplay between ArcA, Crp and EtrA in regulation of respiration of S. oneidensis. To further investigate whether such interplay occurs directly or indirectly, an *in silico* analysis was employed. Previously, we developed ArcA-binding weight matrices using sequences containing an ArcA binding motif derived from transcriptional profiling and EMSA [13,26]. Genome scanning with the matrices revealed 209 operons whose upstream regions contain predicted ArcA binding motifs [13]. Here, we intended to identify the Crp- and EtrA-binding sites in the genome of S. oneidensis with the same strategy but different programs as described in Methods. The analysis with Regulatory Sequence Analysis Tools (RSAT) revealed 214, 254, and 160 genes containing predicted ArcA-, Crp-, and EtrA-binding motifs respectively (Fig. 5 and Table S2, S3, and S4 in Supporting Information). Interestingly, less than a dozen of genes were found to be likely under direct control of ArcA and EtrA. Among the 254 predicted Crp-binding motifs, none of these sites was located within upstream regions of arcA, crp, or etrA, consistent with the observation that expression of these genes were not altered in the Δcrp strain. On the contrary, an

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**Table 4. β-galactosidase activity present in S. oneidensis and E. coli cell extracts.**

| Plasmid               | Features    | β-galactosidase Activity (nmol min⁻¹(mg protein)⁻¹) |
|-----------------------|-------------|---------------------------------------------------|
|                       |             | In E. coli                                        | In S. oneidensis                                    |
| pCM62                 | Plac + lacZ | 42±7/2760±130⁻[a]                                  | 1.7±0.4/2.1±0.5⁻[a]                                 |
| pTP325                | Plac + lacZ | 39±8/2840±125⁻[a]                                  | 424±28/450±37⁻[a]                                   |
| pTP327                | lacZ        | 33±5                                              | 14±4                                               |
| pTP-327-ArcA          | ParcA + lacZ| ND⁻[b]                                            | 236±25                                             |
| pTP-327-Crp           | Pcrp + lacZ | ND⁻[b]                                            | 475±33                                             |
| pTP-327-EtrA          | PetrA + lacZ| ND⁻[b]                                            | 199±21                                             |

⁻[a]Absence/presence of 0.5 mM IPTG.
⁻[b]No data.

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**Figure 3. Construction of a lacZ reporter system.** The full-length lacZ gene obtained by PCR was used to replace lacZs on pCM62. The Plac promoter was then removed from the resultant plasmid pTP325, resulting in the final plasmid pTP327.

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EtrA-binding motif was identified within the upstream region of *crp* while the promoter region of *arcA* contains a binding motif of its own.

ArcA levels in *S. oneidensis*

Although regulation of gene expression at the transcriptional level is indisputably the most important control in bacteria, different post-transcriptional regulation mechanisms have been identified and reported to be crucial in many biological processes [27]. To confirm the promoter activities obtained from the β-galactosidase assay and to investigate whether post-transcriptional control has a significant role in production of ArcA, Crp and EtrA, quantitative immunoblotting analysis was performed to examine the amount of ArcA protein present in these sampled cells was measured as described in Methods. To ensure that signals obtained from the sampled cells were in the linear range, serial dilutions of whole-cell lysates and purified ArcA protein were tested (data not shown). The results indicate that ArcA levels are virtually identical in the wild type and Δcrp strains under either aerobic or anaerobic conditions, consistent with the observations from the β-galactosidase reporter analysis (Fig. 6). However, the amount of ArcA proteins increased only 2.3 ± 0.7 and 1.9 ± 0.5
times in the *ArcA* strain under aerobic and anaerobic conditions, respectively (Fig. 6). Compared to those obtained from the β-galactosidase activity assay, these increases were 3–5 times smaller. An explanation for this difference is that there were 5–7 copies of the plasmid for the transcriptional analysis and only one chromosomal copy for the protein immunoblotting assay.

**Discussion**

*S. oneidensis* MR-1 is an intriguing microorganism in terms of its metabolic pathways. Its genome contains genes encoding many enzymes of mixed acid fermentation but it is not able to ferment [8,28]. Thus the bacteria can only grow by means of aerobic and anaerobic respiration, in which it is renowned for its versatility. [8,28]. Thus the bacterium can only grow by means of aerobic and anaerobic respiration, in which it is renowned for its versatility. Nevertheless, the system is functional and convenient in operation, and more importantly, it is expected to work with other Gram-negative bacteria which lack a *lacZ* analog in the genome. Using the system, an attempt to reveal the interactive control among these three major regulators in *S. oneidensis* was made. One of the most striking findings is that expression of *arcA* was negatively controlled by EtrA given that EtrA has no significant role in regulating anaerobic respiration of *S. oneidensis* [9–10]. Interestingly, both EtrA and ArcA repress *arcA* expression to a similar degree but removal of EtrA does not elicit an obvious phenotype, suggesting that the presence and phosphorylation state of ArcA rather than its net amount is more important for regulation in *S. oneidensis*.

The data from the promoter activity assay gain supports from the bioinformatics analysis in general. Independent expression of *arcA*, *crp*, or *etrA* from Crp is most likely due to the lack of Crp-binding motifs within upstream regions of these genes although other mechanisms such as activation (as in the canonical system) may have a role [37]. By identifying an ArcA-binding motif in close proximity to *arcA*, the in silico analysis reinforces that ArcA represses its own expression through a direct-control mechanism. On the contrary, repression of *arcA* expression by EtrA may possibly be indirect. It is worth noting that more than 60% of top 500 genes under control of either ArcA or Fnr have been shown to be mediated by both regulators directly or indirectly in *E. coli*, suggesting a great deal of functional overlap [38]. In *S. oneidensis*, genes (42) under direct control of both Crp and EtrA (Fig. 5), implicating that Crp and EtrA may be more functionally related. These discrepancies suggest that mechanisms of cellular regulation concluded from *E. coli* may not reflect a general model for bacteria, even not within the same class of *γ*-proteobacteria.

The transcriptional regulatory network in *E. coli* has been extensively studied in recent years and many invaluable insights into this complex process have been obtained [23,39–41]. Although understanding is still far from complete, the finding benefits numerous studies in other microorganisms. However, the results reported previously and presented here demonstrated that *S. oneidensis* differs from *E. coli* profoundly, especially in terms of the function of global regulators. We argue that the differences will lead to a *S. oneidensis* regulatory network which has little in common with the one defined in *E. coli*. To this point, this study is...
particularly of importance by providing useful information for understanding the regulatory network in S. oneidensis.

**Methods**

**Bacterial strains, plasmids, and culture conditions**

A list of all bacterial strains and plasmids used in this study is given in Table 1. *E. coli* and *S. oneidensis* strains under aerobic conditions were grown in Luria-Bertani (LB, Difco, Detroit, MI) medium at 37°C and at room temperature for genetic manipulations, respectively. When needed, the growth medium was supplemented with antibiotics at the following concentrations: ampicillin at 50 µg/ml, gentamycin at 15 µg/ml, and tetracycline at 15 µg/ml.

**Construction of deletion mutants**

A series of deletion strains were constructed for this study. The *arcA* deletion mutant was generated and validated previously and used as the parental strain for constructing subsequent double and triple mutants [13]. Primers used for generating PCR products for mutagenesis are listed in Table S1 in the supplemental material. The mutagenesis process for construction of the *cfr* and *etdA* single deletion mutants, named JZ0624 (*cfr*) and JZ2356 (*etdA*) respectively, followed the procedure described elsewhere [42]. Double mutants JZ90080K-0624 (ArcA-Aept), JZ90080K-2356 (ArcA-Etpt), and JZ0624-2356 (Cfr-Etpt) were generated by repeating the mutagenesis procedure by introduction of a plasmid containing the latter gene deletion structure into the former gene deletion strain. The triple mutant JZ90080K-0524-2356 (ArcA-Etpt-Cfr) was also constructed in this way by introducing the *etdA* deletion structure into the *arcA-*Ept double mutant. The deletion(s) in each strain were verified by PCR and DNA sequencing.

**Physiological characterization of the mutant strains**

M1 defined medium containing 0.02% (w/v) of vitamin-free Casamino Acids and 30 mM lactate was named as M1-L and used in all physiological experiments [43]. Growth of the mutant strains under aerobic or anaerobic conditions was determined by recording growth curves in triplicate with a Bioscreen C microbiology reader (Labsystems Oy, Helsinki, Finland) with the wild-type as the control as described previously [13]. For anaerobic growth, exponential phase cultures grown under aerobic or anaerobic conditions were centrifuged, purged in nitrogen and suspended in fresh medium to approximately ~1×10⁸ cells/ml in an anaerobic glove box. Electron acceptors tested in this study included fumarate (20 mM), nitrate (2 mM), nitrite (1 mM), thiosulfate (3 mM), TMAO (20 mM), and DMSO (20 mM). For electron acceptors containing metals including MnO₂ (5 mM), thiosulfate (3 mM), TMAO (20 mM), and DMSO (20 mM). For electron acceptors containing metals including MnO₂ (5 mM), thiosulfate (3 mM), TMAO (20 mM), and DMSO (20 mM).

**Biochemical methods**

All mutant strains and *S. oneidensis* MR-1 were grown to the late exponential phase either in M1-L under aerobic conditions (OD₆₀₀₉0.6) or in M1-L supplemented with 20 mM TMAO under anaerobic conditions (OD₆₀₀₉0.25). The cells were harvested and then were lysed with lysis buffer (0.25 M Tris/HCl, pH 7.5), 0.5% Trion-X100). Protein concentration was determined with a bicinchoninic acid assay kit with bovine serum albumin (BSA) as a standard according to the manufacturer’s instructions (Pierce Chemical). The amount of heme c was assessed following the procedure described elsewhere [11,44].

**Development of an S. oneidensis lacZ reporter system**

A lacZ reporter system for *S. oneidensis* was developed in this study. The *E. coli lacZ* gene was obtained by PCR amplification with pBlueSTAR-l (Novagen) as the template using primers LacZ-F/R listed in Table S1. The approximately 3 kb PCR product was inserted into the *Rpsl* site of the lacZ gene within the broad-host plasmid pCM62 [18], resulting in pTP325. A short synthetic DNA fragment generated by primers Linker-F/R (Table S1) was used to replace the *Plac* promoter between *Ael* and *HindIII* sites within pTP325. This final plasmid containing promoterless *E. coli lacZ* gene was designated as pTP327.

To construct the *arcA-lacZ*, *crp-lacZ*, and *etdA-lacZ* reporters, the *arcA*, *crp*, and *etdA* promoter DNA fragments were first generated by PCR with primers SO3988-PF/R, SO0624-PF/PR, and SO2356-PF/PR listed in Table S1. These PCR products were then inserted into the *XhoI* and *HindIII* restriction sites of pTP327 individually, resulting in pTP327-3988, pTP327-0624, and pTP327-2356, respectively. After verification by DNA sequencing, the reporter plasmids were moved into each *S. oneidensis* mutant strain used in this study and MR-1 by conjugation. The resulting strains (Table 1) were maintained in LB (aerobic) or LB supplemented with 20 mM lactate, 20 mM TMAO (anaerobic) containing 15 µg/ml tetracycline.

**β-Galactosidase activity assay**

This assay was performed using the High Sensitivity β-Galactosidase Assay Kit from Stratagene. The bacterial cells, collected from aerobic log phase (30°C, OD₆₀₀₉0.3–0.4), were harvested by centrifugation, washed with PBS (phosphate buffered saline), and treated with lysis buffer (0.25 M Tris/HCl, pH 7.5), 0.5% Trion-X100). The resulting soluble protein was collected after centrifugation to remove the insoluble cellular fractions, and subjected to the enzyme assay according to manufacturer’s instructions. The β-galactosidase activity was determined by monitoring color development at 570 nm every minute for 30 min by using a Synergy 2 Multi-Detection Microplate Reader. The protein concentration of the cell lysates was determined using a Bradford assay with BSA as a standard.

**Quantitative Immunoblotting assays for ArcA**

Expression and purification of recombinant *S. oneidensis* His-tagged ArcA protein was performed as described previously [13]. Rabbit polyclonal antibodies against the recombinant ArcA were prepared in accordance with standard protocols provided by the manufacturer (Lampire Biological Laboratories, Pipersville, Pa.) and used for immunoblotting analysis. The cells collected for the β-galactosidase activity assay were used for quantitative immunoblotting assays. For these experiments, cell samples were thawed, washed once with TE buffer (10 mM Tris [pH 8.0], 1 mM EDTA), and resuspended to an...
optical density at 600 nm (OD₆₀₀) of 1.0 in lysis buffer (50 mM Tris [pH 8.0], 1 mM EDTA, 100 mM NaCl). The total protein concentration of the cell lysates was then determined by the bicinchoninic acid assay (Pierce Chemical). Samples were loaded onto SDS-12% polyacrylamide gels and either stained with Coomassie brilliant blue or electrophoretically transferred to nitrocellulose according to manufacturer’s instructions (Bio-Rad). The gels were blotted for 1 h at 50 V using a Criterion blotter (Bio-Rad). An appropriate amount of supernatant was chosen by trial and error for immunoblotting analysis to ensure that the signals observed were not saturated. Images were visualized and quantified with the FluorChem Imaging System in conjunction with AlphaEaseFC software. The linear range for the signal was established by serial dilutions of whole-cell lysates and purified ArcA protein.

CRP and EtaA binding motif analysis

E. coli operons under direct control of CRP and EtaA were derived from reports published previously [45–47]. The promoter regions of obtained operons were subjected to screening for common binding motifs, which was subsequently transformed to a weight matrix using AlignACE [48]. The whole genome was then scanned for putative binding motifs with the weight matrix using RSAT with the default setting [49].

Author Contributions

Conceived and designed the experiments: HG TP JZ. Performed the experiments: HG XW ZKY JC YL. HC. Analyzed the data: HG. Contributed reagents/materials/analysis tools: HG TP JZ. Wrote the paper: HG TP JZ.

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