AMERICAN SOCIETY FOR MICROBIOLOGY | Microbiology Spectrum

| Genetics and Molecular Biology | Research Article

Crp and Arc system directly regulate the transcription of NADH dehydrogenase genes in *Shewanella oneidensis* nitrate and nitrite respiration

Jia-Rong Liu,[1,2] Zhi-Qing Wang,[1,2] Fei-Fei Li,[1,2] Zhen-Kun Li,[1,2] Ming-Chen Wang,[1,2] Na Wang,[1,2] Yu An,[1,2] Xiu-Lan Chen,[2,3] Yu-Zhong Zhang,[1,2,3,4] Hui-Hui Fu[1,2]

AUTHOR AFFILIATIONS See affiliation list on p. 13.

ABSTRACT NADH oxidation by NADH dehydrogenases (NDHs) is crucial for feeding respiratory quinone pool and maintaining the balance of NADH/NAD^+. In the respiratory model organism *Shewanella oneidensis*, which possesses four NDHs, the longstanding notion had been that NDHs were not required under anoxic conditions until recent studies demonstrated their role in extracellular electron transfer. However, the role of each NDH, particularly under anoxic conditions, has not been characterized. Here, we systematically investigated the role of each NDH in aerobic and anaerobic nitrate and nitrite respiration using NDH triple mutants. We corroborated the involvement of NDHs in anaerobic nitrate/nitrite respiration, revealing different repertoires of NDHs employed by *S. oneidensis* in response to electron acceptor (EA) availability. The transcript levels of two *nqrs* were modulated by the EA conversion from nitrate to nitrite. Furthermore, we demonstrated that the global regulators Crp and the Arc system both directly controlled the transcription of four NDHs during nitrate/nitrite respiration. This study confirms the requirement of NDHs for anaerobic nitrate and nitrite respiration and sheds light on the respiratory remodeling mechanism whereby global regulators coordinate NDH genes transcription to adapt to redox-stratified environments.

IMPORTANCE NADH is an important electron source for the respiratory quinone pool. Multiple NADH dehydrogenases (NDHs) are widely present in prokaryotes, indicating the flexibility in NADH oxidation. As a renowned respiratory versatile model strain, *Shewanella oneidensis* possesses four NDHs, encompassing all three types of NDHs, with varying ion-translocating efficiencies. The redundancy of NDHs may confer advantages for *S. oneidensis* to survive and thrive in redox-stratified environments. However, the roles of each NDH, especially in anaerobic respiration, are less understood. Here, we evaluated the role of each NDH in aerobic and anaerobic nitrate/nitrite respiration. We found that the conversion of electron acceptor from nitrate to nitrite triggered the changes in the transcriptional levels of NDH genes, and global regulators Crp and the Arc system were involved in these processes. These findings elucidate the mechanism of the respiratory chain remodeling at the NADH oxidation step in response to different electron acceptors.

KEYWORDS NADH dehydrogenases, respiratory chain, global regulator, *Shewanella oneidensis*

Members of genus *Shewanella*, belonging to γ-proteobacteria, are known for their ability to respire a diverse range of electron acceptors (EAs), including oxygen, nitrate, nitrite, fumarate, dimethylsulfoxide, trimethylamine *N*-oxide, iron, manganese oxides, and electrodes in microbial fuel cells (1–3). Extensive studies have characterized the respiratory chains from quinol pool to terminal EAs using the model strain

Editor Artem S. Rogovsky, Michigan State University, East Lansing, Michigan, USA

Address correspondence to Hui-Hui Fu, fuhuihui@ouc.edu.cn.

Jia-Rong Liu and Zhi-Qing Wang contributed equally to this article. Auther order was determined by a comprehensive evaluation of workload percentage and innovative contributions.

The authors declare no conflict of interest.

Received 18 December 2024
Accepted 31 March 2025
Published 16 May 2025

Copyright © 2025 Liu et al. This is an open-access article distributed under the terms of the Creative Commons Attribution 4.0 International license.

Shewanella oneidensis MR-1 (2, 4–12). However, the upstream of the respiratory chains, where electrons are transferred into the quinone pool, remains poorly understood.

NADH is an important electron carrier in respiratory chains. It must be oxidized into NAD^+ by NADH dehydrogenase (NDH) to feed electrons into the respiratory quinone pool and maintain the balance of NADH/NAD^+, which is crucial for cell survival (13–16). Unlike mammals, which contain only one Nuo in their mitochondrial electron transport chain, prokaryotes often possess multiple NDHs (14, 15, 17–22). The genome of *S. oneidensis* MR-1 encodes four NDHs: one proton-pumping Nuo ($4H^+/2e^-$), two sodium-pumping Nqrs ($2Na^+/2e^-$), and one "uncoupling" Ndh (14–16, 18, 23, 24). Among these, Nuo is the most efficient, as the transfer of 2 e^- from NADH to quinone is coupled with the translocation of 4 H^+ across the inner membrane, contributing to both ATP production and membrane potential maintenance (13, 25, 26). This redundancy of NDHs may facilitate the survival of *S. oneidensis* MR-1 by optimizing its respiratory chains in redox-stratified environments.

Respiratory chain remodeling at the NADH oxidation step has been reported in several prokaryotes (13, 14, 27–31). Duhl et al. (32) recently demonstrated that either Nuo or Nqr1 is essential for aerobic growth in *S. oneidensis* MR-1 by generating the single and double NDH mutants. Subsequent study revealed that the aerobic growth defect of the Δ*nuo*Δ*nqr1* mutant may derive from TCA cycle inhibition caused by an elevated NADH/NAD^+ ratio (25). Nevertheless, the specific roles of individual NDHs in aerobic respiration are still unclear. Under anoxic conditions, *S. oneidensis* MR-1 primarily generates ATP through substrate-level phosphorylation via respiratory chains, with anaerobic lactate catabolism being independent of NADH-dependent enzymes, such as NDHs and pyruvate dehydrogenase (33–37). However, Hirose et al. (38) recently reported that the deletion of all four NDH complexes (ΔNDH mutant) almost completely abolished the ability of current generation of *S. oneidensis* MR-1 in the presence of a high-potential electrode (+0.5 V) and significantly reduced nitrate and MnO_2 reduction capabilities, while fumarate respiration remained unaffected. Following this study, Madsen et al. (26) demonstrated Nuo and Nqr1—previously identified as essential for aerobic growth (32)—also contribute to extracellular electron transfer using either anode (+0.5 V) or Fe^{3+}-NTA as EAs. These results collectively demonstrate the involvement of NDHs in anaerobic respiration of certain EAs.

Transcriptomic analyses of *S. oneidensis* have revealed the transcriptional changes of NDH genes depending on growth conditions (39, 40). These NDH genes are predicted to belong to the regulons of global regulators including Crp, HexR, and EtrA (41–44). Crp, essential for anaerobic respiration in *S. oneidensis* MR-1, is required for growth on diverse EAs (45, 46). The *crp* mutation significantly reduces transcription of key anaerobic respiration genes, including the nitrate reductase gene operon *nap*, nitrite reductase gene *nrfA,* and the two-component system *narPQ* (47). Crp-binding sites are predicted in promoter regions of these genes as well as in the DMSO reductase operon (*dms*) and *cymA*, which encodes a quinol oxidase shared by multiple anaerobic respiratory pathways (48). Notably, potential Crp-binding sites have been identified in the promoter regions of *nuoA* and *nqrA1* (42), suggesting that the globally regulatory effect of Crp may also derive from its regulation on NADH oxidation. The Arc system, a global respiratory regulator that senses the redox state of inner membrane quinone (49), plays critical roles in both aerobic and anaerobic DMSO respiration in *S. oneidensis* (42, 47, 50, 51). Despite sharing 73.6% amino acid sequence identity with *E. coli* Fnr—a master regulator of the aerobic-anaerobic metabolism switch—extensive experimental evidence demonstrates that EtrA exerts no significant regulatory influence on respiratory pathways in *S. oneidensis* MR-1 (42, 52). An ArcA-binding site is predicted in the *dms* operon, and the *arcA* mutant showed reduced *dms* expression (51). Recent study demonstrates that ArcA directly regulates the transcription of *nuo* during electrode respiration (38), promoting us to investigate whether the Arc system regulates NDHs during respiration on the other EAs.

Nitrate and nitrite serve as common EAs for *S. oneidensis* MR-1 via respiratory nitrate ammonification (Fig. 1) (5, 6, 53). The impaired growth of ΔNDH mutant under nitrate-reducing conditions suggests the involvement of NDHs in nitrate and nitrite respiration (38). Notably, the electron transport components from the quinol pool to terminal reductases are identical for both nitrate and nitrite reduction (5, 6). Therefore, the differences in NDH mutants must stem from the absence of certain NDH(s) rather than electron transfer efficiency or preference. The most intriguing feature of respiratory nitrate ammonification in *S. oneidensis* is that nitrite reduction does not occur until nitrate is thoroughly consumed (6). This inherent EA conversion during complete nitrate respiration makes *S. oneidensis* an ideal model for investigating respiratory chain remodeling mechanisms in response to different EAs.

This study aimed to elucidate the respiratory remodeling mechanism at the NADH oxidation step during anaerobic nitrate and nitrite respiration in the model strain *S. oneidensis*. To this end, NDH triple mutants (each remaining only one NDH) were generated and characterized under aerobic and anaerobic nitrate and nitrite respiration conditions. Transcriptional analysis showed that NDH genes responded to the change of available EAs, especially the two *nqrs* exhibited opposing transcriptional responses during the EA conversion from nitrate to nitrite. Furthermore, we demonstrated that both Crp and the Arc system directly regulate the transcription of all four NDHs during nitrate and nitrite respiration. This study characterized the roles of individual NDHs under aerobic and anaerobic conditions and elucidated the mechanism by which the global regulators Crp and Arc coordinate NDH transcription in response to the availability of EAs in the environments.

MATERIALS AND METHODS

Strains, media and culture conditions

Bacterial strains and plasmids used in this study are listed in Table S1. Routinely, *Escherichia coli* and *S. oneidensis* strains were grown in Lysogeny broth (LB) at 37°C and 30°C, respectively, for genetic manipulation. When appropriate, the growth medium was supplemented with the following: 2,6-diaminopimelic acid (DAP), 0.3 mM; gentamicin,

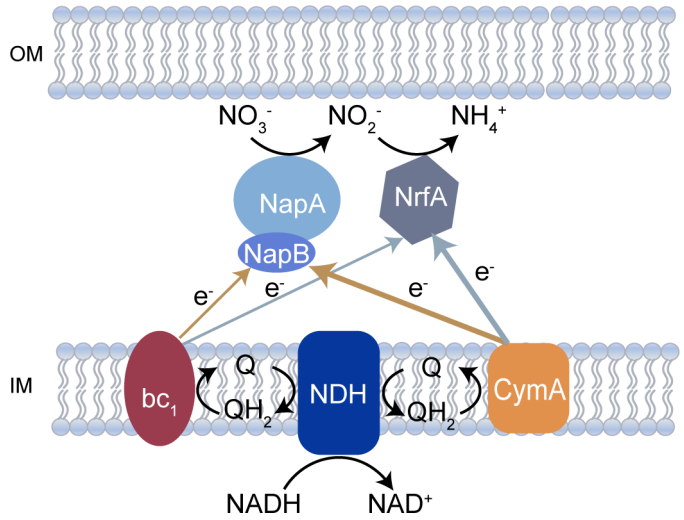


FIG 1 Nitrate and nitrite respiratory chains in *S. oneidensis* MR-1. Electron transfer pathways are color-coded based on respiratory conditions: brown arrows indicate the preferred electron flow during nitrate respiration; light slate gray arrows represent the electron flow during nitrite respiration. Thick arrows denote the primary electron transport mediated by CymA from the quinol pool to nitrate/nitrite reductases, while thin arrows illustrate secondary electron transfer via the cytochrome bc_1 complex. OM, outer membrane; IM, inner membrane.

15 µg/mL; kanamycin, 50 µg/mL; ampicillin, 50 µg/mL. All growth experiments utilized the defined MS minimal medium with lactate added to a final concentration of 20 mM as electron donor, which has been widely used for physiological characterization of *S. oneidensis* MR-1 under both oxic and anoxic conditions (54–56). For anaerobic growth, exponential-phase cultures initially grown aerobically were centrifuged and inoculated into fresh defined MS medium [KCl, 1.34 mM; NaH_2PO_4, 5 mM; Na_2SO_4, 0.7 mM; NaCl, 52 mM; piperazine-*N*,*N*′-bis(2-ethanesulfonic acid) (PIPES), 3 mM; NH_4Cl, 28 mM; sodium lactate, 20 mM; $MgSO_4$, 1 mM; $CaCl_2$, 0.27 mM; and $FeCl_2$, 3.6 µM, pH 7.0] in the Hungate tubes to an OD_{600} of ~0.01. The cultures were purged with nitrogen for ~1 h in the anaerobic chamber until the oxygen content dropped below 5 ppm (57). EAs used in this study included nitrate (5 mM), nitrite (5 mM), and fumarate (20 mM).

In-frame deletion and complementation

In-frame deletion strains were constructed using the *att*-based fusion PCR method, as described previously (58). In brief, the flanking fragments of the target gene were amplified by PCR with *attB*-containing primers and linked by a second round of PCR (Table S2). The fused fragment was cloned into plasmid pHGM01 (58) using the Gateway BP Clonase II enzyme mix (Invitrogen) following the manufacturer's instruction and maintained in *E. coli* WM3064. The resultant plasmid was transferred into *S. oneidensis* via conjugation: Donor cells (*E. coli* WM3064) and recipient cells (*S. oneidensis* MR-1) were separately grown in LB medium to an OD_{600} ≈ 0.4–0.6, mixed at a 2:1 (vol/vol) ratio and spotted onto LB agar plates supplemented with 0.3 mM DAP. The correct conjugants were selected by gentamycin resistance and verified by PCR. Subsequently, verified conjugants were cultured in LB broth lacking NaCl and plated onto LB agar containing 10% (wt/vol) sucrose. Target gene-deletion mutants were screened for gentamicin sensitivity and sucrose resistance, followed by confirmation through DNA sequencing. For the generation of Δ*nuoN*Δ*ndh*Δ*nqrF1*, conjugation and the following screening procedures were performed anaerobically with fumarate and lactate supplemented.

For genetic complementation, the target genes were amplified from the genomic DNA of *S. oneidensis* MR-1 using specific primers (Table S2) and subsequently cloned into the plasmid pHGE-P_{tac} under the control of isopropyl β-D-1-thiogalactopyranoside (IPTG)-inducible P_{tac} promoter (59). The complementation vector was transferred into the corresponding mutant strain via conjugation and verified by PCR and sequencing.

Analysis of the nitrate and nitrite utilization abilities

Strains cultured in defined MS medium with 5 mM nitrate or nitrite as the sole EA under anoxic conditions were sampled at indicated time points. Nitrate and nitrite concentrations in the culture supernatants were quantified using ion chromatography (IC) as previously described (6). The concentrations of nitrite in culture were also measured by a modified Griess assay (60). In all assays, a nitrite standard curve was generated for each assay, and culture samples were diluted appropriately if necessary.

Cell density measurement

The wild-type and four triple mutant strains were anaerobically cultured in defined MS medium with nitrite (5 mM) as the sole EA for 78 h. Cells were harvested and fixed using 5% (vol/vol) glutaraldehyde and then kept in the dark for 20 min. Cell densities were measured using a Guava EasyCyte HT flow cytometer equipped with Guava InCyte v3.1 software, following the instrument configuration described by He et al. (61). After staining with SYBR Green I (Solarbio) for 40 min at room temperature in the dark, cells were serially diluted. An appropriate dilution (1:100, yielding 10^5–10^6 cells/mL) was selected for final cell density calculations.

Real-time qPCR analysis

For aerobic RT-qPCR, bacterial cultures were pre-grown overnight in LB medium, then inoculated into fresh LB medium and incubated at 30°C to OD_{600} ~ 0.6. Cells were subsequently harvested for total RNA extraction. To anaerobic RT-qPCR, bacteria were pre-cultured in LB medium to the mid-exponential phase, centrifuged, and transferred into nitrogen-purged defined MS medium with 20 mM lactate as the electron donor and 5 mM nitrate or nitrite as the sole electron acceptor, respectively. Cells were incubated under anoxic conditions for 2 hours before RNA extraction unless otherwise noted. To assess the transcriptional response of NDH genes to EA conversion, wild-type *S. oneidensis* MR-1 was cultured anaerobically in defined MS medium with 5 mM nitrate as the sole EA and harvested at indicated time points. Total RNA was extracted using the RNeasy Mini Kit (Qiagen, Germany) following the manufacturer's instructions and transcribed into cDNA using a PrimeScript RT reagent Kit with gDNA Eraser (TaKaRa, Japan). The qPCR was performed with specific primers (Table S2) using SYBR Premix Ex Taq (TaKaRa, Japan). Relative gene expression levels were normalized to the housekeeping gene *recA* using the $2^{-\Delta\Delta Ct}$ method.

Gene cloning and protein expression and purification

The *crp* and *arcA* genes were amplified from the *S. oneidensis* MR-1 genome using primers listed in Table S2. The *crp* gene was cloned into the pMAL-c4x vector (NEB, England) to generate an N-terminal maltose-binding protein (MBP)-tagged Crp, while *arcA* was cloned into the pET22b vector (Novagen, Germany) to generate a C-terminal 6 × His-tagged ArcA. For microscale thermophoresis (MST) assays, an additional 6 × His tag was introduced at the C-terminus of Crp via PCR to facilitate the fluorescent labeling (see MST-binding assay section). MBP-Crp and His-tagged ArcA were heterologously expressed in *E. coli* BL21(DE3) by induction with 0.5 mM IPTG. Cells were harvested via centrifugation and resuspended in lysis buffer [50 mM Tris-HCl, 100 mM NaCl, 0.5% (vol/vol) glycerol, pH 8.0]. Cells were disrupted by pressure crusher. MBP-Crp was purified by affinity chromatography using a maltose-column (Cytiva, Sweden), whereas ArcA was purified using a Ni^{2+}-NTA column (GE Healthcare, America). The eluents of MBP-Crp and ArcA were further fractionated by gel filtration on a Superdex G200 column (GE Healthcare, America). As a negative control, the MBP tag alone was amplified from pMAL-c4x vector and purified as MBP-Crp.

Electrophoretic mobility shift assays

EMSAs were performed with purified MBP-Crp or ArcA and biotinylated DNA probes (10 nM) in binding buffer [2 mM EDTA, 20 mM KCl, 0.5 mM dithiothreitol (DTT), 4% (wt/vol) Ficoll-400, pH 8.0] with 2 µg poly (dI-dC) as a nonspecific competitor (62). The promoter regions of NDH operons were amplified using a specific primer set, with the forward primer 5′-end labeled with biotin (Table S2). For reactions involving MBP-Crp, 10 µM cyclic adenosine monophosphate (cAMP) was added to the mixture. To rule out nonspecific interactions between the MBP tag and DNA probes, a control reaction containing purified MBP (instead of MBP-Crp) was included and electrophoresed on the same non-denaturing polyacrylamide gel. Prior to DNA-binding assays, ArcA was phosphorylated *in vitro* using established protocols (5, 50). Competitive EMSAs were conducted by adding a 50-fold molar excess of unlabeled DNA probe to the reaction mixture.

Microscale thermophoresis-binding assay

MBP-Crp (cAMP-activated) or phosphorylated ArcA were labeled with the Large Volume Protein Labeling Kit RED-Tris-NTA 2nd Generation (NanoTemper Technologies GmbH). DNA ligands were serially diluted in a range of concentration and incubated with labeled proteins at 25°C in binding buffer containing 1 × PBS (pH 7.4) and 0.05% (vol/vol) Tween-20. Reaction mixtures were loaded into MonolithTM NT.115 Series capillaries

(NanoTemper Technologies GmbH) and analyzed by MST with the following parameters: 60% LED power and "medium" MST power. The MST data were fit in the MO.Affinity Analysis software to yield the K_d values.

RESULTS

Characterization of NDH mutants in aerobic respiration

To characterize the function of each NDH, triple NDH knockout strains were generated, which only retained one functional NDH. We made in-frame deletion of an essential gene of each NDH operon, *nuoN* (SO_1009), *ndh* (SO_3517), *nqrF1* (SO_1108), and *nqrF2* (SO_0907), as Duhl et al. (32) did. This generated four single mutants, six double mutants, and four triple mutants. Notably, the Δ*nuoN*Δ*ndh*Δ*nqrF1* mutant could only be obtained under anoxic conditions with fumarate as the EA, reminiscent of the aerobic growth defect observed in ΔNDH mutant (38). This implies a negligible role of Nqr2 in aerobic respiration. Among these single and double mutants, only Δ*nuoN*Δ*nqrF1* exhibited severe growth defects in minimal medium with lactate as the sole carbon and energy source under oxic conditions (Fig. S1A and B), consistent with Duhl et al. (32). When any three NDHs were knocked out simultaneously, pronounced growth defects appeared under oxic conditions (Fig. 2). Among them, the growth rate of Δ*nuoN*Δ*ndh*Δ*nqrF1* was lowest (μ = 0.092 ± 0.005 h^{-1}), implying the least contribution of Nqr2 in aerobic NADH oxidation. Meanwhile, Δ*nuoN*Δ*ndh*Δ*nqrF2* had the most robust growth (μ = 0.121 ± 0.009 h^{-1}) compared with the other triple mutants (Δ*nuoN*Δ*ndh*Δ*nqrF1*: μ = 0.092 ± 0.005 h^{-1}; Δ*nuoN*Δ*nqrF1*Δ*nqrF2*: μ = 0.119 ± 0.007 h^{-1}; Δ*ndh*Δ*nqrF1*Δ*nqrF2*: μ = 0.115 ± 0.005 h^{-1}), implying that Nqr1 was the predominant NDH in aerobic respiration. This is consistent with the much higher transcript level of *nqrA1* compared with the other NDH genes under oxic conditions (Fig. S1C). Sodium-pumping NDHs are conserved across *Shewanella* spp., whereas proton-pumping NDHs are sporadically distributed (Table S3). This suggests that Nqrs serve as primary aerobic NDHs in most *Shewanella* members despite that Nuo is more efficient. It is interesting to study the advantage or necessity to remain Nuo in some *Shewanella* strains.

Nitrate and nitrite respiration of NDH triple mutants under anoxic conditions

S. oneidensis employs a two-step nitrate respiration pathway: nitrate is initially reduced to nitrite, which can also serve as an EA of *S. oneidensis*, and only when nitrate is thoroughly consumed does the reduction of nitrite to ammonium occur (Fig. 1) (5, 6).

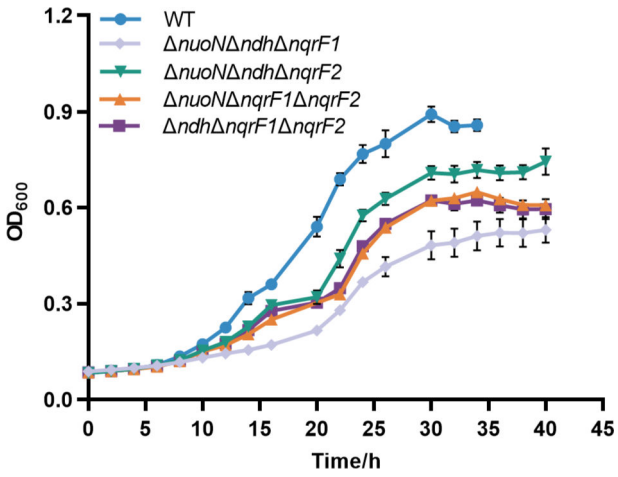


FIG 2 Aerobic growths of wild type and NDH triple mutants. Growth of wild-type *S. oneidensis* MR-1, Δ*nuoN*Δ*ndh*Δ*nqrF1*, Δ*nuoN*Δ*ndh*Δ*nqrF2*, Δ*nuoN*Δ*nqrF1*Δ*nqrF2,* and Δ*ndh*Δ*nqrF1*Δ*nqrF2* strains in defined MS medium with lactate (20 mM) as sole electron donor in aerobic condition. The error bar represents the standard deviation of triplicate experiments. All experiments were carried out at least three times.

Previous studies indicate that ΔNDH exhibited impaired nitrate utilization (38), implying the involvement of NDHs in anaerobic nitrate respiration. However, the observed growth defect of ΔNDH could derive from functional deficiencies in either or both steps of the pathway. To determine the exact roles of NDHs in each step, we quantified the concentrations of nitrate during anaerobic cultivation of wild-type MR-1 and four NDH triple mutant strains in minimal medium containing nitrate (5 mM) as the sole EA (50). All four triple mutants displayed nitrate consumption rates comparable to wild type (Fig. 3A), implying that the absence of any three NDHs barely impairs the nitrate respiration to nitrite. There are two possible explanations for this: one is that any NDH alone is sufficient to meet NADH oxidation requirements for nitrate to nitrite reduction; the other is that nitrate reduction to nitrite proceeds independently of NDHs. Therefore, ΔNDH is needed to distinguish between these possibilities.

When nitrate was replaced by nitrite, all triple mutants exhibited impaired nitrite utilization, with Δ*ndh*Δ*nqrF1*Δ*nqrF2* showing the most severe defect (Fig. 3B). Consistently, the maximum biomass yield supported by nitrite respiration was lowest in Δ*ndh*Δ*nqrF1*Δ*nqrF2* compared to the wild-type and other triple mutants (Fig. S2). These results demonstrate that the most efficient NDH, Nuo, contributes minimally to nitrite respiration. This physiologically distinction aligns with the markedly lower biomass yield from anaerobic nitrite respiration compared to aerobic respiration. Since nitrite is generally cytotoxic, we cannot exclude the possibility that Nuo is more sensitive to nitrite toxicity than other NDHs. Collectively, our findings indicate that NDHs are involved in anaerobic nitrate respiration at least during the nitrite reduction step, and the relative contributions of individual NDHs varied significantly depending on the EAs utilized by *S. oneidensis*.

Transcript levels of NDH genes respond to the conversion of EA from nitrate to nitrite

To determine whether *S. oneidensis* remodels its respiratory chain by regulating NDHs expression at the transcriptional level in response to different EAs, we measured the mRNA abundance of the first gene of each NDH operon in wild-type MR-1 during complete nitrate respiration. Mid-log phase cells (OD_{600} ~ 0.6) were inoculated into defined M5 medium with nitrate (5 mM) as the sole EA and harvested at designated

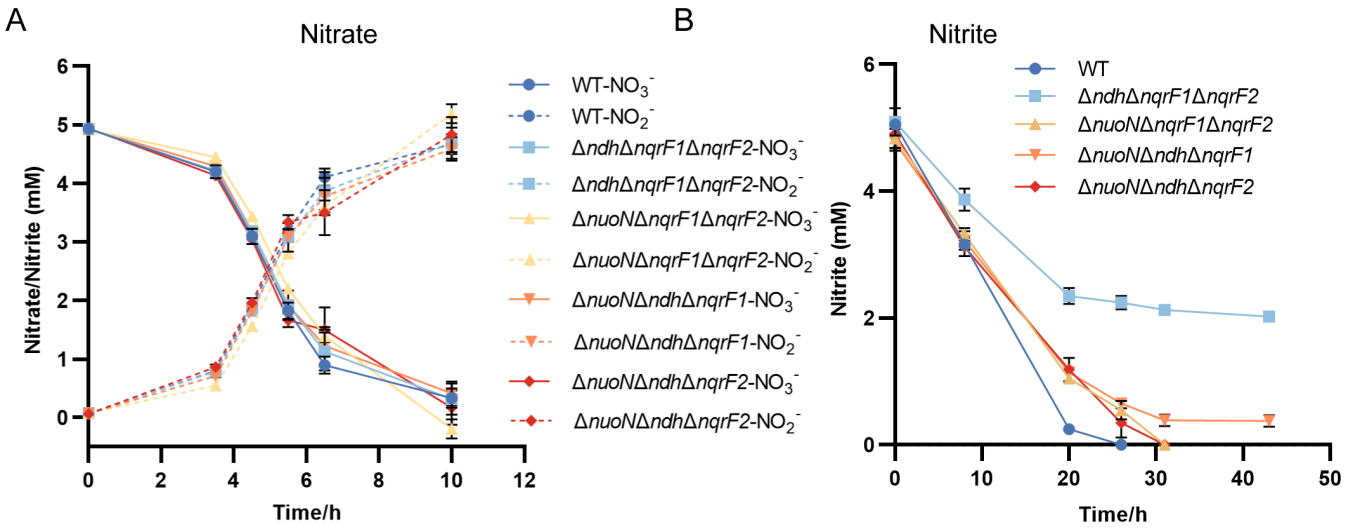


FIG 3 Consumption of nitrate and nitrite by wild type and NDH triple mutants under anoxic condition. Nitrate and nitrite utilization of wild-type *S. oneidensis* MR-1, Δ*nuoN*Δ*ndh*Δ*nqrF1*, Δ*nuoN*Δ*ndh*Δ*nqrF2*, Δ*nuoN*Δ*nqrF1*Δ*nqrF2*, and Δ*ndh*Δ*nqrF1*Δ*nqrF2* strains in defined MS medium with lactate (20 mM) as sole electron donor and 5 mM nitrate (A) or nitrite (B) as sole EA, respectively, under anoxic conditions. Cultures were sampled at indicated times for nitrate and nitrite detection. The concentrations of nitrate and nitrite reduced from nitrate were shown simultaneously when nitrate was the EA. The error bar represents standard deviation of triplicate experiments. All experiments were carried out at least three times.

time points. Nitrite (the nitrate reduction product) accumulated to ~5 mM, indicating complete nitrate reduction, before being subsequently consumed (Fig. 4).

Transcriptional analysis revealed that *nqrA1* transcription increased moderately (~1.6-fold) during nitrite respiration compared to nitrate respiration (Fig. 4A), while *nqrA2* transcription progressively decreased (up to ~5-fold) during the conversion of EA from nitrate to nitrite (Fig. 4B). These results imply that *S. oneidensis* differentially regulates the two *nqrs* depending on EA availability, potentially as an energy-conserving strategy. The transcript level of *nuoA* was gradually increased (~2.5-fold) with the accumulation of nitrite (Fig. 4C). However, the transcript levels of *nuoA* did not respond to nitrite concentration (Fig. S3). The *nuoA* transcription could not explain the limited contribution of Nuo to nitrite respiration yet (Fig. 3B). In contrast to these dynamic changes, *ndh* transcription remained relatively stable throughout the respiration process (Fig. 4D), implying the constitutive expression of the only one "non-ion-pumping" NDH in *S. oneidensis*. Collectively, these results demonstrate that the conversion of available EA significantly alters the transcript levels of most NDHs (except for *ndh*), which may contribute to respiratory remodeling at the NADH oxidation step.

Crp and Arc system coordinate in modulating the transcription of two *nqrs*

The divergent transcriptional responses of the two homologous *nqr* operons to the conversion of EA from nitrate to nitrite promoted investigation into potential regulatory mechanisms. We focused on the global respiration regulators Crp and the Arc two-component system in *S. oneidensis*. Mutant strains Δ*crp* and Δ*arcA* (encoding the response regulator of the Arc system) (63) were generated to assess their roles in nitrate and nitrite utilization. Consistent with previous studies (42, 47), Δ*crp* barely utilized nitrate and nitrite (Fig. S4A and B). While Δ*arcA* exhibited only minor defects in nitrate and nitrite utilization (Fig. S4C and D), suggesting a limited role in nitrate and nitrite respiration.

To explore the involvement of Crp and ArcA in the divergent responses of two *nqrs*, the transcript levels of two *nqrAs* were measured in wild-type and mutant strains. Mid-log phase cultures (OD_{600} ~0.6) were transferred into anaerobic medium with either nitrate or nitrite supplemented as sole EA and harvested after 2 h (ensuring nitrate was the authentically utilized EA in nitrate-supplemented medium). The *nqrA1* transcript levels were increased 2.4- to 3.1-fold in Δ*crp* compared to wild type, and this enhancement was reduced in the presence of cloned *crp* (Fig. 5A). Conversely, *nqrA2* transcription decreased up to 8.3- to 11.1-fold in Δ*crp* compared to wild type, which

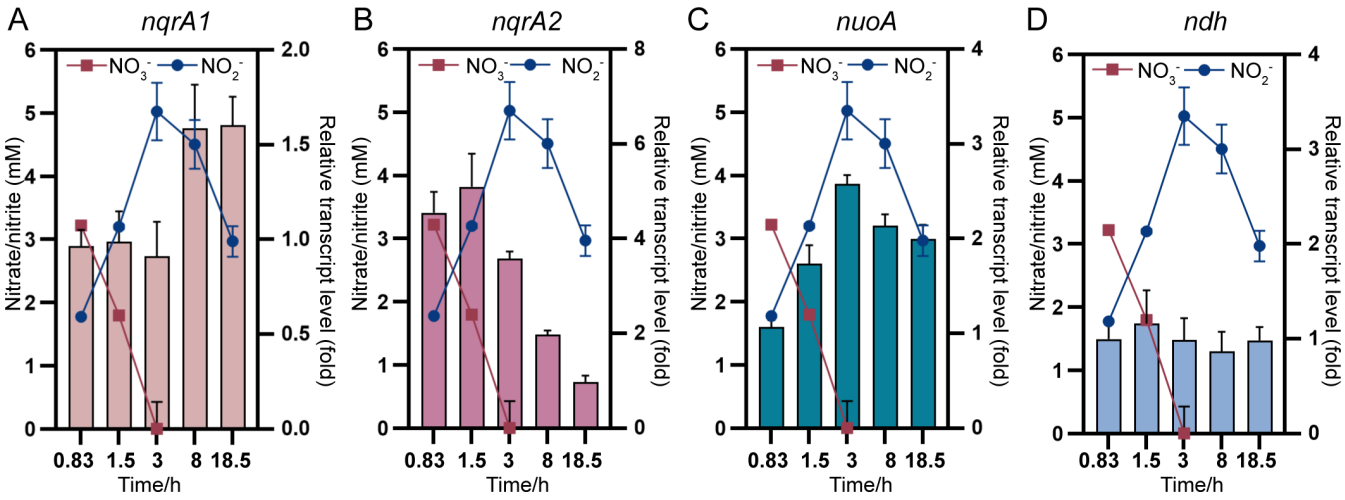


FIG 4 Changes in relative transcript levels of NDH genes in response to EA conversion from nitrate to nitrite under anoxic conditions. Relative transcript levels of *nqrA1* (A), *nqrA2* (B), *nuoA* (C), and *ndh* (D) in wild-type strain grown in defined MS medium with nitrate (5 mM) as sole EA in anaerobic condition. The nitrate and nitrite concentration curves were embedded to indicate the EA used at indicated sampling time points. The error bar represents the standard deviation of triplicate experiments. All experiments were carried out at least three times.

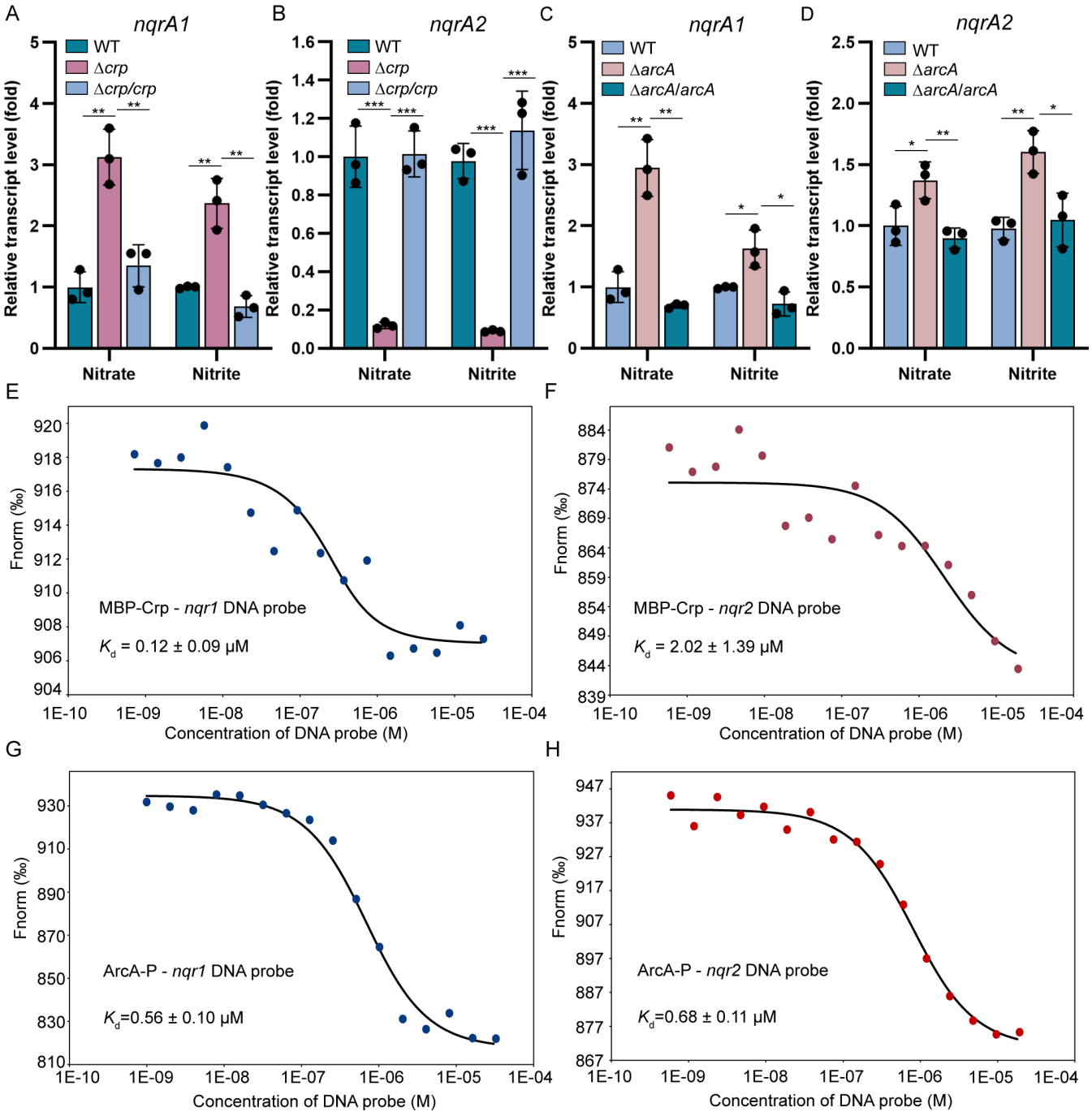


FIG 5 Crp and ArcA directly regulate the transcription of *nqr1* and *nqr2*. (A, B) Relative transcript levels of *nqrA1* (A) and *nqrA2* (B) in wild-type, Δ*crp,* and its genetically complemented strain Δ*crp/crp* grown in defined MS medium with 5 mM nitrate or nitrite as EA under anoxic conditions. The error bar represents standard deviation of triplicate experiments. (C, D). Relative transcript levels of *nqrA1* (C) and *nqrA2* (D) in wild-type, Δ*arcA,* and its genetically complemented strain Δ*arcA/arcA* grown in defined MS medium with 5 mM nitrate or nitrite as EA under anoxic conditions. The error bar represents standard deviation of triplicate experiments. (E, F) MST analysis of MBP-Crp binding to *nqr1* promoter probe (E) or *nqr2* promoter probe (F). (G, H) MST analysis of ArcA-P binding to *nqr1* promoter probe (G) or *nqr2* promoter probe (H). A two-sided Student's *t*-test was used to assess statistically significant differences ($^{***}P < 0.001$; $^{**}P < 0.01$; $^{*}P < 0.05$). All experiments were carried out at least three times.

was fully restored by cloned *crp* (Fig. 5B). This demonstrates Crp's dual regulatory role as a repressor of *nqr1* but activator of *nqr2*. The deletion of *arcA* produced more modest effects, causing only ~1.5-fold expression changes except for *nqrA1* in nitrate-reducing

condition (~3 fold) (Fig. 5C and D). This indicates ArcA acts as a repressor for two *nqrs*; however, its regulatory function is far less pronounced than that of Crp in modulating *nqr2* transcription.

To explore the regulatory mechanisms further, we purified recombinant MBP-tagged Crp and His-tagged ArcA. MST analysis demonstrated that cAMP-activated MBP-Crp had K_d values of 0.12 ± 0.09 µM and 2.02 ± 1.39 µM for *nqr1* promoter probe and *nqr2* promoter probe, respectively (Fig. 5E and F). Control experiments confirmed the specific nature of these interaction, as MBP alone could not bind to either probe (Fig. S5A and B). Consistently, the EMSA shift bands were detected for both probes incubated with cAMP-activated MBP-Crp but not with MBP control (Fig. S5C and D). These data, collectively, indicate that Crp directly regulates the transcription of two *nqrs*. Parallel analyses with phosphorylated ArcA (ArcA-P) revealed similar DNA-binding capability. MST yielded K_d values of 0.56 ± 0.10 µM and 0.68 ± 0.11 µM for ArcA-P binding to *nqr1* and *nqr2* promoters, respectively (Fig. 5G and H). Corresponding EMSA experiments showed specific protein-DNA complexes that were effectively competed by 50-fold excess unlabeled probes (Fig. S5E and F). These results indicate that both Crp and ArcA regulate the transcription of two *nqrs* by directly binding to their promoter regions.

Crp and ArcA also directly regulate the transcription of *nuo* and *ndh*

Although transcript levels of *nuo* and *ndh* did not respond to changes in EA under complete nitrate respiration (Fig. 4C and D), we further examined the regulatory roles of Crp and ArcA on these operons. Under nitrate-reducing condition, the transcript level of *nuoA* in Δ*crp* increased moderately (~2.4-fold) compared to wild-type cells, an enhancement abolished by complementation with *crp* (Fig. 6A). However, under nitrite-reducing condition, the regulatory effect of *crp* on the *nuoA* was not significant (Fig. 6A). Potential Crp-binding site had been predicted in the promoter region of *nuoA* (42), implying direct regulation by Crp. To validate this, we performed MST and EMSA using the purified Crp and the *nuo* promoter DNA probe, following methods described for *nqrs*. As expected, interaction was observed between the *nuo* promoter probe and MBP-Crp, but not with MBP alone (Fig. 6B; Fig. S6A and B). These results indicate that Crp directly regulates the transcription of *nuo*.

Although ArcA has been reported to directly regulate *nuo* transcription during electrode respiration in *S. oneidensis* (38) and *E. coli* (64; Park et al. 2013), no significant regulatory effect of ArcA on *nuo* was observed under either nitrate- or nitrite-reducing condition (Fig. S6C). Nevertheless, consistent with previous findings (38), MST and EMSA confirmed interaction between ArcA-P and the *nuo* promoter probe (Fig. 6C; Fig. S6D). Thus, we speculate that the regulatory role of Δ*arcA* may be suppressed by Crp, which dominates transcriptional control during nitrate and nitrite respiration.

The absence of *crp* caused derepression of *ndh* under nitrate-reducing conditions (Fig. 6D). Like the other NDH genes, direct binding of MBP-Crp to the *ndh* promoter region was verified by both MST and EMSA (Fig. 6E; Fig. S6E and F), implying the involvement of *ndh* in the Crp regulon. In contrast, the deletion of *arcA* had negligible effects on the transcript levels of *ndh* in both nitrate- and nitrite-reducing conditions (Fig. S6G). Intriguingly, interactions between ArcA-P and the *ndh* promoter probe were still detected (Fig. 6F; Fig. S6H). Taken together, these results demonstrate that *nuo* and *ndh* are primarily regulated by Crp in nitrate and nitrite respiration, and the Arc system is also potentially involved.

DISCUSSION

The long-standing assumption that NDHs are dispensable under anoxic conditions in *S. oneidensis* has been challenged by recent evidence demonstrating their role in high-potential electrode respiration (38). The *S. oneidensis* MR-1 genome encodes four NDHs spanning three distinct families, highlighting its flexibility in NADH oxidation. To date, only two studies have reported phenotypes associated with the double mutant

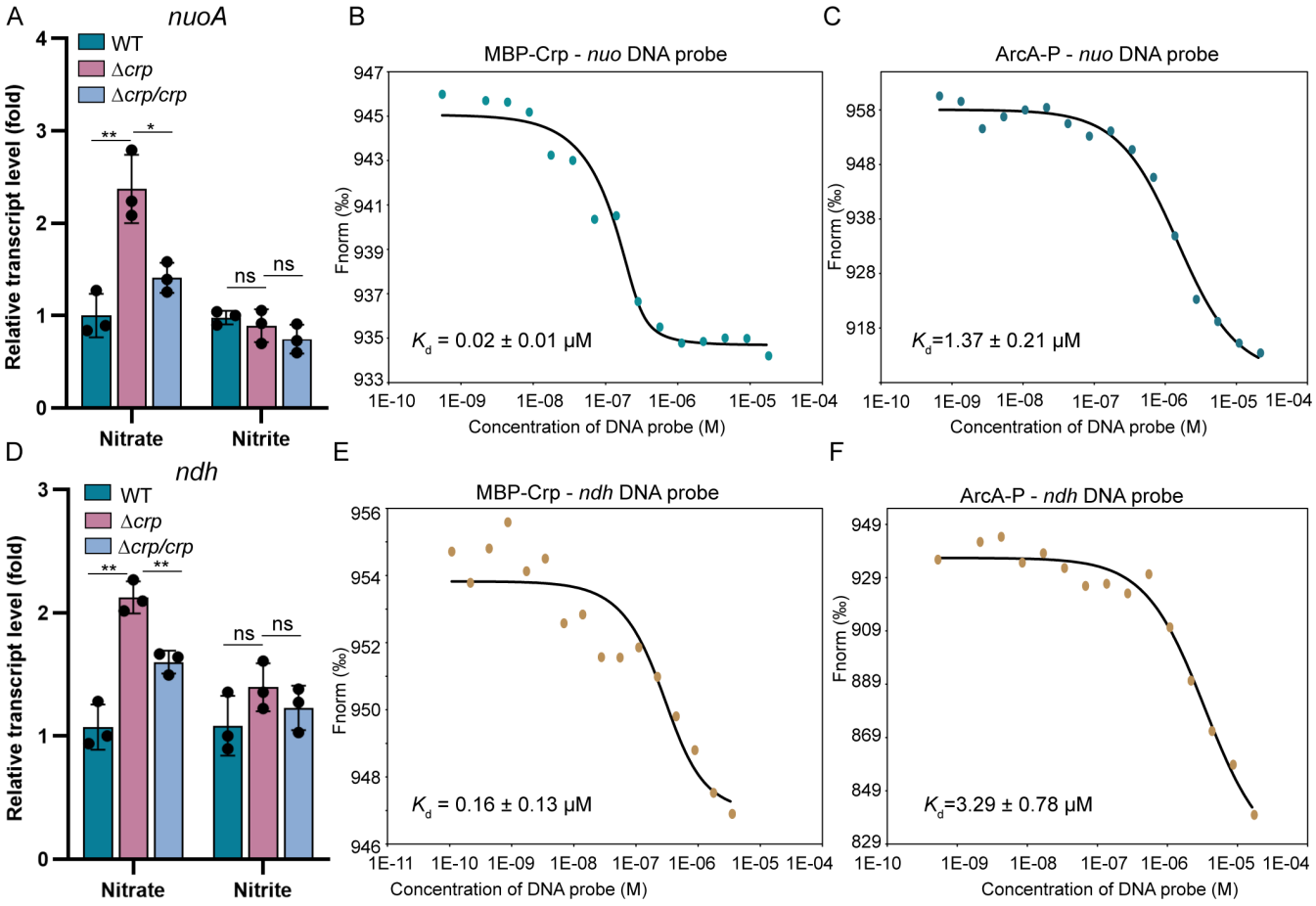


FIG 6 Crp and ArcA directly regulate the transcription of *nuo* and *ndh*. (A) Relative transcript levels of *nuoA* in wild-type, Δ*crp,* and its genetically complemented strain Δ*crp*/*crp* grown in defined MS medium with 5 mM nitrate or nitrite as EA under anoxic conditions. The error bar represents standard deviation of triplicate experiments. (B) MST analysis of MBP-Crp binding to *nuo* promoter probe. (C) MST analysis of ArcA-P binding to *nuo* promoter probe. (D) Relative transcript levels of *ndh* in wild-type, Δ*crp,* and its genetically complemented strain Δ*crp*/*crp* grown in defined MS medium with 5 mM nitrate or nitrite as EA under anoxic conditions. The error bar represents standard deviation of triplicate experiments. (E) MST analysis of MBP-Crp binding to *ndh* promoter probe. (F) MST analysis of ArcA-P binding to *ndh* promoter probe. A two-sided Student's *t*-test was used to assess statistically significant differences ($^{**}P < 0.01$; $^{*}P < 0.05$; ns, $P > 0.05$). All experiments were carried out at least three times.

Δ*nuoN*Δ*nqrF1*: impaired aerobic growth in minimal medium (32) and reduced current generation with high-potential electrodes (26). Notably, an apparent discrepancy exists between the Δ*nuoN*Δ*nqrF1* phenotype reported by Duhl et al. (32) and those observed in our study. While Duhl et al. described a complete loss of aerobic growth in M5 minimal medium, we observed only a significant growth defect under analogous conditions using defined MS medium (Fig. S1B). This divergence may stem from methodological differences, particularly in culture volume (300 µL vs 1 mL/50 mL) and medium composition (MS vs M5).

Critically, the individual contributions of NDHs have not been characterized yet, particularly under anoxic conditions. To address this gap, we generated four NDH triple mutants and systematically evaluated their roles in aerobic and anaerobic nitrate and nitrite respiration. Our findings reveal dynamic respiratory chain remodeling at the NADH oxidation step in response to changes in EA availability. Furthermore, leveraging stepwise nitrate respiration, we identified the four NDH genes as members of the Crp and Arc regulons, which contribute to the remodeling of respiratory chains at the transcriptional level.

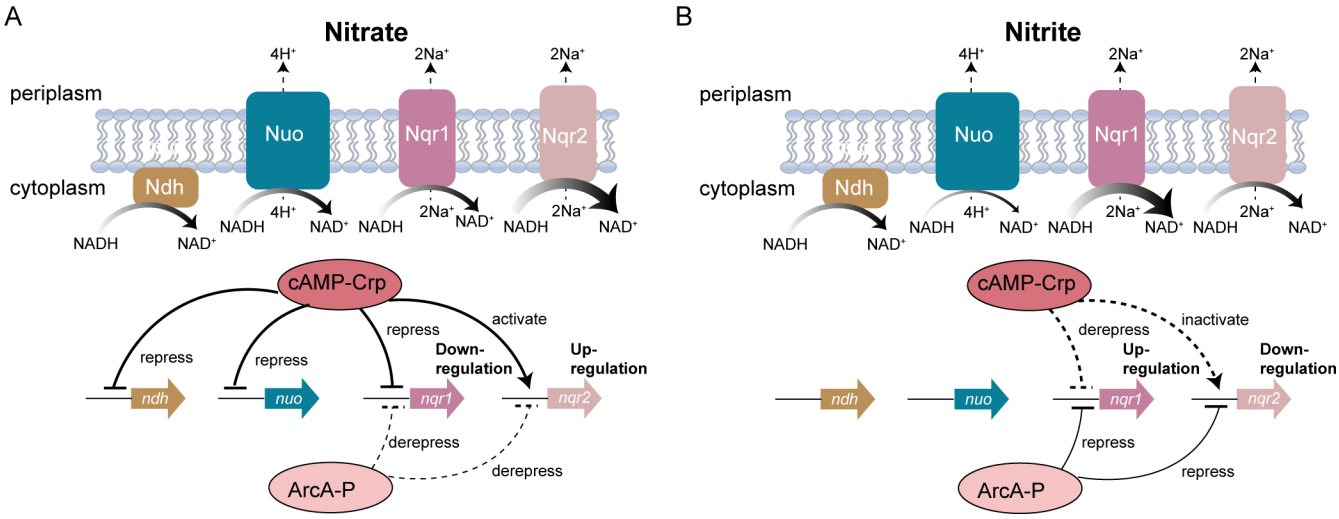


FIG 7 Proposed regulatory model of Crp and ArcA on the NDH genes in response to EA conversion from nitrate to nitrite. (A) When nitrate serves as EA, cAMP-activated Crp (cAMP-Crp) binds to the promoter region of *nqr1* operon as a repressor and while acting as an activator at the *nqr2* operon promoter. Although phosphorylated ArcA (ArcA-P) derepresses transcription of both *nqr* operons, its derepression effect on *nqr1* is counteracted by cAMP-Crp-mediated repression. The coordinated regulation by Crp and ArcA collectively downregulates *nqr1* expression and upregulates *nqr2* expression. Additional, cAMP-Crp binds to the promoter regions of *nuo* and *ndh*, repressing their transcription. (B) When nitrite serves as EA, cAMP-Crp derepresses the transcription of *nqr1* while inactivating *nqr2*. ArcA-P represses both *nqr* operons; however, its repression effect on *nqr1* is counteracted by cAMP-Crp-mediated derepression. This regulatory interplay results in upregulated *nqr1* expression and downregulated *nqr2* expression during nitrite respiration. Notably, no significant transcriptional regulation of *nuo* and *ndh* by Crp or ArcA was observed in nitrite respiration.

Sodium-pumping NDHs are prevalent in the *Shewanella* genus. Here, we demonstrate that the two *nqr* operons exhibit opposing transcriptional responses to EA conversion from nitrate to nitrite, a process critically governed by Crp. When nitrate serves as the EA, Crp directly represses *nqr1* while activating *nqr2* (Fig. 7A). Conversely, during nitrite utilization, Crp derepresses *nqr1* and inactivates *nqr2* (Fig. 7B). This regulatory switch ensures that only one *nqr* is robustly expressed during EA conversion in complete nitrate respiration. It is interesting to study the regulatory mechanism of Crp to discriminate the two homologous *nqr* operons.

In addition, the Arc regulatory system exerts modest repressive effects on both *nqr* operons during both nitrate and nitrite respiration (Fig. 7A and B), consistent with its canonical role in catabolic gene repression (64). Nitrate, a preferred high-redox-potential EA (5, 64), likely elevates the oxidized form of inner membrane quinones (65), mimicking conditions observed in the presence of oxygen (49) or high-potential electrode (38). This redox shift is sensed by the Arc system, triggering dephosphorylation of ArcA. Dephosphorylated ArcA derepresses both *nqr* operons (Fig. 7A). In contrast, when nitrite, a relatively low-redox-potential EA, is utilized, phosphorylated ArcA represses the transcription of both *nqrs* (Fig. 7B).

Nitrate serves as a preferred EA for bacteria due to its high standard redox potential. Our results showed that nitrate can be efficiently reduced by the *S. oneidensis* wild type and NDH triple mutants. In contrast, Δ*nuo*NΔ*nqrF1* exhibits significant defects in the respiration of high redox potential electrode and Fe(III)-NTA (26), processes that depend on the extracellular electron transfer pathway. These findings suggest that the redox potential of the utilized EA may not determine the preference for NDHs. The produced nitrite is a low redox potential EA. During nitrite respiration, all NDH triple mutants showed varying degrees of respiratory defects while retaining nitrite reduction capability, indicating that all four NDHs are involved in nitrite respiration. Interestingly, our results show that Nuo—the most efficient NDH—contributes least to nitrite respiration (Fig. 3B). Even though both Crp and the Arc system potentially regulate the transcription of *nuo*, this regulatory pattern cannot fully explain the limited contribution

of Nuo to nitrite respiration. Notably, *S. oneidensis* possesses only one "uncoupling" Ndh. Our findings reveal that *ndh* also belongs to both the Crp and Arc regulons; however, its transcriptional level remains relatively stable during both nitrate and nitrite respiration. This consistent expression pattern suggests that *ndh* may play a crucial role in dissipating excess reducing power during anaerobic respiration.

In conclusion, this study demonstrates distinct NDH repertoire preferences during respiration of different EAs in *S. oneidensis*. Our findings corroborate that NDHs participate not only in extracellular insoluble EA respiration (38) but also in anaerobic respiration of periplasmic soluble EAs. The transcriptional change triggered by EA conversion mediates respiratory chain remodeling at the NADH oxidation step in *S. oneidensis*. Specifically, global regulators Crp and the Arc system coordinately control NDHs transcription. Crp plays a dominant role, particularly in mediating opposing transcriptional regulation between two *nqrs*. Our findings reveal that beyond their established regulation of terminal reductase genes, the global regulators Crp and Arc system also orchestrate upstream respiratory network complexity through NADH oxidation modulation. These regulatory mechanisms likely enable *Shewanella* species to dynamically remodel their respiratory chains for optimal survival in redox-stratified environments.

ACKNOWLEDGMENTS

This work was supported by the National Science Foundation of China (42176156, 92251303, 42276102, 42076229), the National Key R & D program of China (2022YFC2807500, 2021YFA0909600), the Fundamental Research Funds for the Central Universities (202172002), Major Scientific and Technological Innovation Project (MSTIP) of Shandong Province (2019JZZY010817), and the Taishan Scholars Program of Shandong Province, China (tspd20181203, tsqn202306092).

AUTHOR AFFILIATIONS

[1]MOE Key Laboratory of Evolution and Marine Biodiversity, Frontiers Science Center for Deep Ocean Multispheres and Earth System & College of Marine Life Sciences, Ocean University of China, Qingdao, China
[2]Laboratory for Marine Biology and Biotechnology, Qingdao Marine Science and Technology Center, Qingdao, China
[3]State Key Laboratory of Microbial Technology, Marine Biotechnology Research Center, Shandong University, Qingdao, China
[4]Joint Research Center for Marine Microbial Science and Technology, Shandong University and Ocean University of China, Qingdao, China

AUTHOR ORCIDs

Xiu-Lan Chen http://orcid.org/0000-0003-2991-3631
Yu-Zhong Zhang http://orcid.org/0000-0002-2017-1005
Hui-Hui Fu http://orcid.org/0000-0001-8101-863X

AUTHOR CONTRIBUTIONS

Jia-Rong Liu, Data curation, Formal analysis, Investigation, Methodology | Zhi-Qing Wang, Data curation, Investigation, Methodology | Fei-Fei Li, Data curation, Investigation | Zhen-Kun Li, Data curation, Investigation | Ming-Chen Wang, Data curation, Investigation | Na Wang, Data curation, Investigation | Yu An, Data curation, Investigation | Xiu-Lan Chen, Writing – review and editing | Yu-Zhong Zhang, Funding acquisition, Supervision, Writing – review and editing | Hui-Hui Fu, Conceptualization, Funding acquisition, Methodology, Project administration, Writing – original draft, Writing – review and editing

ADDITIONAL FILES

The following material is available online.

Supplemental Material

Supplemental material (Spectrum03324-24-s0001.docx). Tables S1 to S3; Fig. S1 to S6.

REFERENCES

1. Fredrickson JK, Romine MF, Beliaev AS, Auchtung JM, Driscoll ME, Gardner TS, Nealson KH, Osterman AL, Pinchuk G, Reed JL, Rodionov DA, Rodrigues JLM, Saffarini DA, Serres MH, Spormann AM, Zhulin IB, Tiedje JM. 2008. Towards environmental systems biology of *Shewanella*. Nat Rev Microbiol 6:592–603. https://doi.org/10.1038/nrmicro1947
2. Myers CR, Nealson KH. 1988. Bacterial manganese reduction and growth with manganese oxide as the sole electron acceptor. Science 240:1319–1321. https://doi.org/10.1126/science.240.4857.1319
3. Shi L, Dong H, Reguera G, Beyenal H, Lu A, Liu J, Yu H-Q, Fredrickson JK. 2016. Extracellular electron transfer mechanisms between microorganisms and minerals. Nat Rev Microbiol 14:651–662. https://doi.org/10.1038/nrmicro.2016.93
4. Cordova CD, Schicklberger MFR, Yu Y, Spormann AM. 2011. Partial functional replacement of CymA by SirCD in *Shewanella oneidensis* MR-1. J Bacteriol 193:2312–2321. https://doi.org/10.1128/JB.01355-10
5. Fu H., Jin M, Ju L, Mao Y, Gao H. 2014. Evidence for function overlapping of CymA and the cytochrome bc1 complex in the *Shewanella oneidensis* nitrate and nitrite respiration. Environ Microbiol 16:3181–3195. https://doi.org/10.1111/1462-2920.12457
6. Gao Haichun, Yang ZK, Barua S, Reed SB, Romine MF, Nealson KH, Fredrickson JK, Tiedje JM, Zhou J. 2009. Reduction of nitrate in *Shewanella oneidensis* depends on atypical NAP and NRF systems with NapB as a preferred electron transport protein from CymA to NapA. ISME J 3:966–976. https://doi.org/10.1038/ismej.2009.40
7. Kotloski NJ, Gralnick JA. 2013. Flavin electron shuttles dominate extracellular electron transfer by *Shewanella oneidensis*. MBio 4:e00553-12. https://doi.org/10.1128/mBio.00553-12
8. Marsili E, Baron DB, Shikhare ID, Coursolle D, Gralnick JA, Bond DR. 2008. *Shewanella* secretes flavins that mediate extracellular electron transfer. Proc Natl Acad Sci USA 105:3968–3973. https://doi.org/10.1073/pnas.0710525105
9. Myers CR, Myers JM. 1993. Ferric reductase is associated with the membranes of anaerobically grown *Shewanella putrefaciens* MR-1. FEMS Microbiol Lett 108:15–22. https://doi.org/10.1111/j.1574-6968.1993.tb06066.x
10. Myers C.R, Myers JM. 1997. Cloning and sequence of cymA, a gene encoding a tetraheme cytochrome c required for reduction of iron(III), fumarate, and nitrate by *Shewanella putrefaciens* MR-1. J Bacteriol 179:1143–1152. https://doi.org/10.1128/jb.179.4.1143-1152.1997
11. Myers CR, Nealson KH. 1990. Respiration-linked proton translocation coupled to anaerobic reduction of manganese(IV) and iron(III) in *Shewanella putrefaciens* MR-1. J Bacteriol 172:6232–6238. https://doi.org/10.1128/jb.172.11.6232-6238.1990
12. Schwalb C, Chapman SK, Reid GA. 2003. The tetraheme cytochrome CymA is required for anaerobic respiration with dimethyl sulfoxide and nitrite in *Shewanella oneidensis*. Biochemistry 42:9491–9497. https://doi.org/10.1021/bi034456f
13. Duarte M, Ferreira C, Khandpur GK, Flohr T, Zimmermann J, Castro H, Herrmann JM, Morgan B, Tomás AM. 2021. *Leishmania* type II dehydrogenase is essential for parasite viability irrespective of the presence of an active complex I. Proc Natl Acad Sci USA 118:e2103803118. https://doi.org/10.1073/pnas.2103803118
14. Gyan S, Shiohira Y, Sato I, Takeuchi M, Sato T. 2006. Regulatory loop between redox sensing of the NADH/NAD(+) ratio by Rex (YdiH) and oxidation of NADH by NADH dehydrogenase Ndh in *Bacillus subtilis*. J Bacteriol 188:7062–7071. https://doi.org/10.1128/JB.00601-06
15. Kerscher S, Dröse S, Zickermann V, Brandt U. 2007. The three families of respiratory NADH dehydrogenase. Results Probl Cell Differ 45:185–222. https://doi.org/10.1007/400_2007_028
16. Refojo PN, Sena FV, Calisto F, Sousa FM, Pereira MM. 2019. The plethora of membrane respiratory chains in the phyla of life. Adv. Microb:331–414. https://doi.org/10.1016/bs.ampbs.2019.03.002
17. Kostner D, Luchterhand B, Junker A, Volland S, Daniel R, Büchs J, Liebl W, Ehrenreich A. 2015. The consequence of an additional NADH dehydrogenase paralog on the growth of Gluconobacter oxydans DSM3504. Appl Microbiol Biotechnol 99:375–386. https://doi.org/10.1007/s00253-014-6069-9
18. Melo AMP, Bandeiras TM, Teixeira M. 2004. New insights into type II NAD(P)H:quinone oxidoreductases. Microbiol Mol Biol Rev 68:603–616. https://doi.org/10.1128/MMBR.68.4.603-616.2004
19. Nies SC, Dinger R, Chen Y, Wordofa GG, Kristensen M, Schneider K, Büchs J, Petzold CJ, Keasling JD, Blank LM, Ebert BE. 2020. Systems analysis of NADH dehydrogenase mutants reveals flexibility and limits of *Pseudomonas taiwanensis* VLB120's metabolism. Appl Environ Microbiol 86:e03038-19. https://doi.org/10.1128/AEM.03038-19
20. Wackwitz B, Bongaerts J, Goodman SD, Unden G. 1999. Growth phase-dependent regulation of nuoA-N expression in *Escherichia coli* K-12 by the fis protein: upstream binding sites and bioenergetic significance. Mol Gen Genet 262:876–883. https://doi.org/10.1007/s004380051153
21. Weinstein EA, Yano T, Li L-S, Avarbock D, Avarbock A, Helm D, McColm AA, Duncan K, Lonsdale JT, Rubin H. 2005. Inhibitors of type II NADH:menaquinone oxidoreductase represent a class of antitubercular drugs. Proc Natl Acad Sci USA 102:4548–4553. https://doi.org/10.1073/pnas.0500469102
22. Matsushita K, Ohnishi T, Kaback HR. 1987. NADH-ubiquinone oxidoreductases of the *Escherichia coli* aerobic respiratory chain. Biochemistry 26:7732–7737. https://doi.org/10.1021/bi00398a029
23. Barquera B. 2014. The sodium pumping NADH:quinone oxidoreductase (Na+the sodium pumping NADH:quinone oxidoreductase (Na. J Bioenerg Biomembr 46:289–298. https://doi.org/10.1007/s10863-014-9565-9
24. Walker JE. 1992. The NADH:ubiquinone oxidoreductase (complex I) of respiratory chains. Quart Rev Biophys 25:253–324. https://doi.org/10.1017/S003358350000425X
25. Duhl KL, TerAvest MA. 2019. *Shewanella oneidensis* NADH dehydrogenase mutants exhibit an amino acid synthesis defect. Front Energy Res 7:116. https://doi.org/10.3389/fenrg.2019.00116
26. Madsen CS, TerAvest MA. 2019. NADH dehydrogenases Nuo and Nqr1 contribute to extracellular electron transfer by *Shewanella oneidensis* MR-1 in bioelectrochemical systems. Sci Rep 9:14959. https://doi.org/10.1038/s41598-019-51452-x
27. Marino M, Hoffmann T, Schmid R, Möbitz H, Jahn D. 2000. Changes in protein synthesis during the adaptation of *Bacillus subtilis* to anaerobic growth conditions. Microbiology (Reading) 146 (Pt 1):97–105. https://doi.org/10.1099/00221287-146-1-97
28. Yagi T. 1993. The bacterial energy-transducing NADH-quinone oxidoreductases. Biochim Biophys Acta 1141:1–17. https://doi.org/10.1016/0005-2728(93)90182-f
29. Tran QH, Bongaerts J, Vlad D, Unden G. 1997. Requirement for the proton-pumping NADH dehydrogenase I of *Escherichia coli* in respiration of NADH to fumarate and its bioenergetic implications. Eur J Biochem 244:155–160. https://doi.org/10.1111/j.1432-1033.1997.00155.x
30. Vilchèze C, Weinrick B, Leung LW, Jacobs WR. 2018. Plasticity of *Mycobacterium tuberculosis* NADH dehydrogenases and their role in virulence. Proc Natl Acad Sci USA 115:1599–1604. https://doi.org/10.1073/pnas.1721545115
31. Ye RW, Tao W, Bedzyk L, Young T, Chen M, Li L. 2000. Global gene expression profiles of Bacillus subtilis grown under anaerobic conditions. J Bacteriol 182:4458–4465. https://doi.org/10.1128/JB.182.16.4458-4465.2000
32. Duhl KL, Tefft NM, TerAvest MA, Drake HL. 2018. Shewanella oneidensis MR-1 utilizes both sodium- and proton-pumping NADH dehydrogenases during aerobic growth. Appl Environ Microbiol 84:e00415-18. https://doi.org/10.1128/AEM.00415-18

33. Hunt KA, Flynn JM, Naranjo BN, Shikhare ID, Gralnick JA. 2010. Substrate-level phosphorylation is the primary source of energy conservation during anaerobic respiration of *Shewanella oneidensis* strain MR-1. J Bacteriol 192:3345–3351. https://doi.org/10.1128/JB.00090-10
34. Kane AL, Brutinel ED, Joo H, Maysonet R, VanDrisse CM, Kotloski NJ, Gralnick JA. 2016. Formate metabolism in *Shewanella oneidensis* generates proton motive force and prevents growth without an electron acceptor. J Bacteriol 198:1337–1346. https://doi.org/10.1128/JB.00927-15
35. Pinchuk Grigoriy E, Geydebrekht OV, Hill EA, Reed JL, Konopka AE, Beliaev AS, Fredrickson JK. 2011. Pyruvate and lactate metabolism by Shewanella oneidensis MR-1 under fermentation, oxygen limitation, and fumarate respiration conditions. Appl Environ Microbiol 77:8234–8240. https://doi.org/10.1128/AEM.05382-11
36. Pinchuk Grigory E, Rodionov DA, Yang C, Li X, Osterman AL, Dervyn E, Geydebrekht OV, Reed SB, Romine MF, Collart FR, Scott JH, Fredrickson JK, Beliaev AS. 2009. Genomic reconstruction of *Shewanella oneidensis* MR-1 metabolism reveals a previously uncharacterized machinery for lactate utilization. Proc Natl Acad Sci USA 106:2874–2879. https://doi.org/10.1073/pnas.0806798106
37. Tang YJ, Meadows AL, Kirby J, Keasling JD. 2007. Anaerobic central metabolic pathways in *Shewanella oneidensis* MR-1 reinterpreted in the light of isotopic metabolite labeling. J Bacteriol 189:894–901. https://doi.org/10.1128/JB.00926-06
38. Hirose A, Kasai T, Aoki M, Umemura T, Watanabe K, Kouzuma A. 2018. Electrochemically active bacteria sense electrode potentials for regulating catabolic pathways. Nat Commun 9:1083. https://doi.org/10.1038/s41467-018-03416-4
39. Beliaev AS, Klingeman DM, Klappenbach JA, Wu L, Romine MF, Tiedje JM, Nealson KH, Fredrickson JK, Zhou J. 2005. Global transcriptome analysis of *Shewanella oneidensis* MR-1 exposed to different terminal electron acceptors. J Bacteriol 187:7138–7145. https://doi.org/10.1128/JB.187.20.7138-7145.2005
40. Rosenbaum MA, Bar HY, Beg QK, Segrè D, Booth J, Cotta MA, Angenent LT. 2012. Transcriptional analysis of *Shewanella oneidensis* MR-1 with an electrode compared to Fe(III)citrate or oxygen as terminal electron acceptor. PLoS One 7:e30827. https://doi.org/10.1371/journal.pone.0030827
41. Cruz-García C, Murray AE, Rodrigues JLM, Gralnick JA, McCue LA, Romine MF, Löffler FE, Tiedje JM. 2011. Fnr (EtrA) acts as a fine-tuning regulator of anaerobic metabolism in *Shewanella oneidensis* MR-1. BMC Microbiol 11:64. https://doi.org/10.1186/1471-2180-11-64
42. Gao Haichun, Wang X, Yang ZK, Chen J, Liang Y, Chen H, Palzkill T, Zhou J. 2010b. Physiological roles of ArcA, Crp, and EtrA and their interactive control on aerobic and anaerobic respiration in *Shewanella oneidensis*. PLoS One 5:e15295. https://doi.org/10.1371/journal.pone.0015295
43. Leyn SA, Li X, Zheng Q, Novichkov PS, Reed S, Romine MF, Fredrickson JK, Yang C, Osterman AL, Rodionov DA. 2011. Control of proteobacterial central carbon metabolism by the HexR transcriptional regulator: a case study in *Shewanella oneidensis*. J Biol Chem 286:35782–35794. https://doi.org/10.1074/jbc.M111.267963
44. Novichkov PS, Kazakov AE, Ravcheev DA, Leyn SA, Kovaleva GY, Sutormin RA, Kazanov MD, Riehl W, Arkin AP, Dubchak I, Rodionov DA. 2013. RegPrecise 3.0--a resource for genome-scale exploration of transcriptional regulation in bacteria. BMC Genomics 14:745. https://doi.org/10.1186/1471-2164-14-745
45. Saffarini DA, Schultz R, Beliaev A. 2003. Involvement of cyclic AMP (cAMP) and cAMP receptor protein in anaerobic respiration of *Shewanella oneidensis*. J Bacteriol 185:3668–3671. https://doi.org/10.1128/JB.185.12.3668-3671.2003
46. Charania MA, Brockman KL, Zhang Y, Banerjee A, Pinchuk GE, Fredrickson JK, Beliaev AS, Saffarini DA. 2009. Involvement of a membrane-bound class III adenylate cyclase in regulation of anaerobic respiration in *Shewanella oneidensis* MR-1. J Bacteriol 191:4298–4306. https://doi.org/10.1128/JB.01829-08
47. Dong Y, Wang J, Fu H, Zhou G, Shi M, Gao H. 2012. A Crp-dependent two-component system regulates nitrate and nitrite respiration in *Shewanella oneidensis*. PLoS One 7:e51643. https://doi.org/10.1371/journal.pone.0051643
48. Gao H, Barua S, Liang Y, Wu L, Dong Y, Reed S, Chen J, Culley D, Kennedy D, Yang Y, He Z, Nealson KH, Fredrickson JK, Tiedje JM, Romine M, Zhou J. 2010a. Impacts of *Shewanella oneidensis* c-type cytochromes on aerobic and anaerobic respiration. Microb Biotechnol 3:455–466. https://doi.org/10.1111/j.1751-7915.2010.00181.x
49. Georgellis D, Kwon O, Lin ECC. 2001. Quinones as the redox signal for the arc two-component system of bacteria. Science 292:2314–2316. https://doi.org/10.1126/science.1059361
50. Gao H., Wang X, Yang ZK, Palzkill T, Zhou J. 2008. Probing regulon of ArcA in *Shewanella oneidensis* MR-1 by integrated genomic analyses. BMC Genomics 9:42. https://doi.org/10.1186/1471-2164-9-42
51. Gralnick JA, Brown CT, Newman DK. 2005. Anaerobic regulation by an atypical Arc system in *Shewanella oneidensis*. Mol Microbiol 56:1347–1357. https://doi.org/10.1111/j.1365-2958.2005.04628.x
52. Maier TM, Myers CR. 2001. Isolation and characterization of a *Shewanella putrefaciens* MR-1 electron transport regulator etrA mutant: reassessment of the role of EtrA. J Bacteriol 183:4918–4926. https://doi.org/10.1128/JB.183.16.4918-4926.2001
53. Cruz-García C, Murray AE, Klappenbach JA, Stewart V, Tiedje JM. 2007. Respiratory nitrate ammonification by *Shewanella oneidensis* MR-1. J Bacteriol 189:656–662. https://doi.org/10.1128/JB.01194-06
54. Fu H., Liu L, Dong Z, Guo S, Gao H. 2018. Dissociation between iron and heme biosyntheses is largely accountable for respiration defects of *Shewanella oneidensis fur* mutants. Appl Environ Microbiol 84:e00039-18. https://doi.org/10.1128/AEM.00039-18
55. Shi M, Wan F, Mao Y, Gao H. 2015. Unraveling the mechanism for the viability deficiency of *Shewanella oneidensis* oxyR Null Mutant. J Bacteriol 197:2179–2189. https://doi.org/10.1128/JB.00154-15
56. Sun W, Xu Y, Liang Y, Yu Q, Gao H. 2023. A novel bacterial sulfite dehydrogenase that requires three *c*-type cytochromes for electron transfer. Appl Environ Microbiol 89:e0110823. https://doi.org/10.1128/aem.01108-23
57. Fu Huihui, Chen H, Wang J, Zhou G, Zhang H, Zhang L, Gao H. 2013. Crp-dependent cytochrome bd oxidase confers nitrite resistance to *Shewanella oneidensis*. Environ Microbiol 15:2198–2212. https://doi.org/10.1111/1462-2920.12091
58. Jin M, Jiang Y, Sun L, Yin J, Fu H, Wu G, Gao H. 2013. Unique organizational and functional features of the cytochrome c maturation system in *Shewanella oneidensis*. PLoS One 8:e75610. https://doi.org/10.1371/journal.pone.0075610
59. Luo Q, Dong Y, Chen H, Gao H. 2013. Mislocalization of rieske protein PetA predominantly accounts for the aerobic growth defect of Tat mutants in *Shewanella oneidensis*. PLoS One 8:e62064. https://doi.org/10.1371/journal.pone.0062064
60. Miranda KM, Espey MG, Wink DA. 2001. A rapid, simple spectrophotometric method for simultaneous detection of nitrate and nitrite. Nitric Oxide 5:62–71. https://doi.org/10.1006/niox.2000.0319
61. He X-Y, Liu N-H, Liu J-Q, Peng M, Teng Z-J, Gu T-J, Chen X-L, Chen Y, Wang P, Li C-Y, Todd JD, Zhang Y-Z, Zhang X-Y. 2023. SAR92 clade bacteria are potentially important DMSP degraders and sources of climate-active gases in marine environments. MBio 14:e0146723. https://doi.org/10.1128/mbio.01467-23
62. Zhang P, Wu L, Zhu Y, Liu M, Wang Y, Cao G, Chen X-L, Tao M, Pang X. 2017. Deletion of MtrA Inhibits Cellular Development of *Streptomyces coelicolor* and Alters Expression of Developmental Regulatory Genes. Front Microbiol 8:2013. https://doi.org/10.3389/fmicb.2017.02013
63. Lassak J, Henche AL, Binnenkade L, Thormann KM. 2010. ArcS, the cognate sensor kinase in an atypical Arc system of *Shewanella oneidensis* MR-1. Appl Environ Microbiol 76:3263–3274. https://doi.org/10.1128/AEM.00512-10
64. Bongaerts J, Zoske S, Weidner U, Unden G. 1995. Transcriptional regulation of the proton translocating NADH dehydrogenase genes (nuoA-N) of Escherichia coli by electron acceptors, electron donors and gene regulators. Mol Microbiol 16:521–534. https://doi.org/10.1111/j.1365-2958.1995.tb02416.x
65. Green J, Paget MS. 2004. Bacterial redox sensors. Nat Rev Microbiol 2:954–966. https://doi.org/10.1038/nrmicro1022