*Shewanella oneidensis* MR-1 utilizes both sodium- and proton-pumping NADH dehydrogenases during aerobic growth

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**Abstract**

*Shewanella oneidensis* MR-1 is a metal reducing bacterium with the ability to utilize many different terminal electron acceptors, including oxygen and solid metal oxides. Both metal oxide reduction and aerobic respiration have been studied extensively in this organism. However, electron transport chain processes upstream of the terminal oxidoreductases have been relatively understudied in this organism, especially electron transfer from NADH to respiratory quinones. Genome annotation indicates that *S. oneidensis* MR-1 encodes four NADH dehydrogenases: a proton-translocating dehydrogenase (Nuo); two sodium ion-translocating dehydrogenases (Nqr1 and Nqr2); and an ‘uncoupling’ dehydrogenase (Ndh), but none of these complexes has been studied. Therefore, we conducted a study specifically focused on the effects of individual NADH dehydrogenase knockouts in *S. oneidensis* MR-1. We observed that two of the single mutant strains, ΔnuoN and ΔnqrF1, exhibited significant growth defects compared with wild type. However, the defects were minor and only apparent under certain growth conditions. Further testing of a double mutant strain, ΔnuoNΔnqrF1, yielded no growth in minimal media under oxic conditions, indicating that Nuo and Nqr1 have overlapping function, but at least one is necessary for aerobic growth. Co-utilization of proton- and sodium ion-dependent energetics has important implications for growth of this organism in environments with variable pH and salinity, including microbial electrochemical systems.

**Importance**

Bacteria utilize a wide variety of metabolic pathways that allow them to take advantage of different energy sources, and to do so with varying efficiency. The efficiency of a metabolic process determines the
growth yield of an organism, or the amount of biomass it produces per amount of substrate consumed. This parameter has important implications in biotechnology and wastewater treatment, where low growth yields are often preferred to minimize production of microbial biomass. In this study, we investigated respiratory pathways containing NADH dehydrogenases with varying efficiency (i.e., the number of ions translocated per NADH oxidized) in the metal-reducing bacterium *Shewanella oneidensis* MR-1. We observed that two different respiratory pathways are used concurrently, and at least one must be functional for growth under oxic conditions.

**Introduction**

*Shewanella oneidensis* MR-1 is a facultative anaerobe with the capability to respire using a wide variety of terminal electron acceptors in the absence of oxygen (1). One of the best-studied aspects of this organism is its use of solid metal oxides and electrodes as terminal electron acceptors (2). *S. oneidensis* MR-1 interacts with solid electron acceptors via the Mtr pathway, which transfers electrons to the acceptor either through direct contact or soluble flavin electron shuttles (3–5). This capability is useful in many different bioelectrochemical technologies (6–9). For example, *S. oneidensis* MR-1 has been engineered to act as a biosensor by linking Mtr expression, and therefore electric current generation, to a chemical signal in the environment (10, 11). ‘Unbalanced fermentation’ has also been developed in *S. oneidensis* MR-1, allowing it to overcome redox imbalance between substrates and products by releasing excess reducing equivalents to an anode electrode via the Mtr pathway (12).

While the Mtr pathway and other terminal oxidoreductases are well-studied, upstream processes that transfer electrons into the respiratory quinol pool are less understood. *S. oneidensis* MR-1 uses a variety of complexes to transfer electrons into the quinol pool, and these may use one of several different electron donors including primary substrates, such as lactate, or electron carriers, such as NADH (13). In *S. oneidensis* MR-1, there has been significant research on lactate dehydrogenases (14, 15), hydrogenases (16, 17), and formate dehydrogenases (18). However, to our knowledge, the NADH dehydrogenases have only been studied incidentally in whole-genome expression profiling, without specific gene deletion or...
biochemical studies. Four NADH dehydrogenases are encoded in the genome of *S. oneidensis* MR-1: one predicted to pump protons (Nuo, SO_1009 to SO_1021), two predicted to pump sodium ions (Nqr1, SO_1103 to SO_1108; Nqr2, SO_0902 to SO_0907), and one predicted to be ‘uncoupling’ that does not translocate ions across the inner membrane (Ndh, SO_3517) (19). We note that the ‘Nqr1’ and ‘Nqr2’ labels are not used consistently across studies and genome databases, but here we refer to them as shown above.

Both sodium-pumping NADH dehydrogenases (Nqr) are found in all sequenced genomes in the *Shewanella* genus, while the proton-pumping NADH dehydrogenase (Nuo) has been found in only a few isolates, including *S. oneidensis* MR-1 (19). The same pattern has been observed with sodium- and proton-dependent flagellar stators, with *S. oneidensis* MR-1 being the only isolate known to contain the proton-dependent MotAB flagellar rotation system, while all sequenced *Shewanella* isolates contain the sodium-dependent PomAB system (20). Although this suggests that the sodium-motive force (SMF) is the major energetic gradient used by *S. oneidensis* MR-1, other studies point to the importance of proton-motive force (PMF). For example, the proton-specific uncoupler carbonyl cyanide m-chlorophenylhydrazone (CCCP) induces biofilm dissolution in *S. oneidensis* MR-1 (21). Further, PMF generation by a light-driven proton pump improves current production and survival of *S. oneidensis* MR-1 in bioelectrochemical systems (22). Finally, we observed with BLAST (23) that the F$_{0}$F$_{1}$ ATP synthase of *S. oneidensis* MR-1 appears to be powered by PMF due to the lack of specific residues necessary for sodium ion transport (24). Of course, PMF and SMF cannot be completely disentangled, because both are composed of two components: membrane potential ($\Delta\Psi$) and concentration gradient of the coupling ion ($\Delta$pH or $\Delta$[Na$^{+}$]). PMF and SMF are further connected by proton-sodium antiporters. The *S. oneidensis* MR-1 genome encodes several Na$^{+}$/H$^{+}$ antiporters: NhaA, NhaB, NhaC, NhaD, and two in the cation:proton antiporter-1 family (25). Therefore, it may be possible for *S. oneidensis* MR-1 to use sodium as the primary ion for respiratory coupling, but convert SMF to PMF through antiporters for ATP synthesis. While this may appear to be an inefficient strategy, a recent review describes several organisms...
that utilize this method of oxidative phosphorylation, indicating that it may have advantages in some environments (26).

While NADH dehydrogenase activities have not been directly studied in *S. oneidensis* MR-1, some basic information about their function can be gleaned from the large amount of transcriptomic data collected for this organism. Several studies have detected significant changes in transcriptional regulation of the NADH dehydrogenases depending on the growth condition. Rosenbaum et al. (27) found upregulation of the proton-pumping NADH dehydrogenase (*nuo*) in electrode-grown biofilms compared with aerobically-grown planktonic cells. In contrast, Beliaev et al. (1) found decreased expression of *nuo* and *nqr2* with oxygen and metal oxide electron acceptors compared to fumarate. These studies show that NADH dehydrogenase expression is dependent on electron acceptor type, but do not yet reveal a clear pattern.

Studies on known *S. oneidensis* MR-1 regulons also help predict when each NADH dehydrogenase is expressed. The regulation of Nqr NADH dehydrogenases appears to be based on carbon source type, energy levels within the cell, and the presence of specific electron acceptors. The *nqr2* operon is regulated by the hexose-dependent HexR regulator, suggesting expression of this NADH dehydrogenase depends on the available carbon source (28). In contrast, *nqr1* is not predicted to be part of the HexR regulon, but rather it is regulated by the cAMP-dependent Crp regulator (29). The Fnr-like regulator EtrA also appears to play a role, leading to increased expression of *nqr2* under anoxic conditions, and increased expression of *nqr1* under oxic conditions (30). Although it is unclear if Nqr1 and Nqr2 differ in function, due to high sequence identity, differential regulation of each dehydrogenase suggests that they serve distinct roles in metabolism.

NADH is one of the most important electron carriers in cellular metabolism and appears to be critical for respiratory activity in *S. oneidensis* MR-1, based on the presence of genes encoding four different NADH dehydrogenases in its genome. However, electron transport from NADH to the quinol pool in *S. oneidensis* MR-1 has been understudied compared with terminal oxidases. To understand how
S. oneidensis MR-1 couples growth to processes such as electric current production and environmental metal cycling, it is essential to understand how PMF and SMF are generated during respiration. Therefore, we have conducted a directed study on the role of each putative NADH dehydrogenase in the S. oneidensis MR-1 genome by generating in-frame deletions to disrupt each complex and studying phenotypes of the mutant strains under oxic conditions. Our strategy was informed by a new analysis of an existing whole-genome fitness profiling dataset.

Results

Mining a whole-genome fitness dataset for NADH dehydrogenase utilization patterns

We analyzed an existing whole-genome fitness profiling dataset from a study by Deutschbauer et al. (31) to gain initial insights into environmental conditions that influence the function of each NADH dehydrogenase encoded in the S. oneidensis MR-1 genome. In the study, a whole-genome transposon mutant library was grown under more than 200 different conditions. Fitness of the mutants was measured by change in abundance of sequence tags throughout the experiment. To focus on specific carbon sources, we plotted fitness scores of NADH dehydrogenase mutant strains in a subset of 55 conditions where the library was grown in minimal medium under oxic or anoxic conditions with lactate or N-acetylglucosamine (NAG) as the substrate (Figure 1). Conditions with differing nitrogen, sulfur, and carbon sources (other than NAG and lactate) were excluded from the subset. Mutants in nuo (proton-dependent) and nqr1 (sodium ion-dependent) genes showed the greatest fitness defects under oxic conditions. This effect was weaker with lactate than with NAG, which is unsurprising considering that lactate oxidation theoretically generates 4 NADH per molecule compared to 13 NADH per molecule for NAG (Figure 2). Mutants with insertions in ndh (uncoupling) and nqr2 (sodium ion-dependent) genes showed greater fitness defects in anoxic conditions compared to oxic conditions. In general, all fitness defects were weak under anoxic conditions, which may be due to the lack of significant TCA cycle activity in S. oneidensis MR-1 when oxygen is absent (32), resulting in less NADH generation from most substrates. Based on this analysis, we focused on oxic conditions, which showed the largest overall fitness
defects for the NADH dehydrogenase mutants in the library. We chose two different carbon sources, sodium D,L-lactate and NAG, to understand the effects of substrates that generate differing amounts of NADH.

Analysis of NADH dehydrogenase mutant growth and metabolism

To study the impact of NADH dehydrogenase activity on growth and metabolism, we created an in-frame deletion in an essential gene in each putative NADH dehydrogenase-encoding operon in *S. oneidensis* MR-1; *nuoN* (SO_1009), *ndh* (SO_3517), *nqrF1* (SO_1108), and *nqrF2* (SO_0907). Deletions were made in the terminal gene in each operon to restrict downstream pleiotropic effects while removing function of each dehydrogenase. We cultured wild type *S. oneidensis* MR-1 (WT) and the NADH dehydrogenase mutants in minimal medium in 24-well plates and measured growth via OD$_{600}$. Our measurements aligned well with observations from the whole-genome fitness analysis, confirming that culturing the mutants using D,L-lactate or NAG has differential effects on growth. The Δ*ndh* and Δ*nqrF2* mutants did not show growth defects with either NAG or D,L-lactate as carbon sources under oxic conditions and therefore were omitted from further study (Figure S1).

Growth studies were repeated using a higher volume in flasks. As predicted by fitness data and growth in 24-well plates, both Δ*nuoN* and Δ*nqrF1* showed growth defects in minimal medium supplemented with 10 mM NAG as the carbon source (Figure 3A). Both mutants had significantly decreased growth rates compared with WT: 0.93 ± 0.05 s$^{-1}$ for WT, 0.76 ± 0.06 s$^{-1}$ for Δ*nuoN*, and 0.81 ± 0.04 s$^{-1}$ for Δ*nqrF1* (Table 1). To better understand the mechanism of the growth defect, the concentrations of substrates and metabolic byproducts in the culture were monitored by HPLC throughout growth. We observed that both mutants consumed significantly less NAG than WT at the 12- (Δ*nqrF1*, $p \leq 0.01$; Δ*nuoN*, $p \leq 0.05$) and 16-hour (Δ*nqrF1*, $p \leq 0.001$; Δ*nuoN*, $p \leq 0.01$) time points (Figure 3B).

Acetate accumulation in cultures of both mutant strains was significantly higher than in cultures of WT at 20 hours of growth ($p \leq 0.01$) although all strains had consumed the excreted acetate by 40 hours (Figure 3C). No other major products were observed.
Flask growth experiments were also performed using 20 mM D,L-lactate as the substrate. Growth rates were not significantly different from WT for either ΔnqrF1 or ΔnuoN (Table 1). However, ΔnqrF1 showed a distinct delay in growth although all cultures were inoculated to the same cell density at the same time (i.e., overall OD600 is significantly lower than WT from 12 to 16 hours). This effect is less pronounced than the growth rate defect with NAG, but was repeatable (Figure 4A, Figure S1). Similar to growth, D,L-lactate consumption and acetate accumulation by the ΔnuoN mutant were essentially indistinguishable from WT. However, the ΔnqrF1 mutant displayed subtle differences from WT. ΔnqrF1 used D,L-lactate more slowly than WT during early logarithmic growth (Figure 4B). However, by 24 hours both WT and ΔnqrF1 both consumed all available lactate (Figure 4B). ΔnqrF1 also accumulated 25% more acetate than WT at 24 hours and had not fully consumed it by 40 hours (Figure 4C).

Because we observed increased acetate accumulation by the mutant strains with both substrates, we hypothesized that they had a decreased capacity to consume acetate as a substrate compared to WT. To determine if the mutant strains exhibited a reduced ability to utilize acetate, we conducted a 24-well growth experiment in minimal medium supplemented with 10 mM acetate (Figure 5). Both mutant strains ΔnuoN and ΔnqrF1 exhibited growth defects compared to WT in this growth condition (Table 1). The WT, ΔnuoN, and ΔnqrF1 grew at rates of $0.55 \pm 0.04$ s$^{-1}$, $0.36 \pm 0.02$ s$^{-1}$ ($p \leq 0.01$), and $0.32 \pm 0.01$ s$^{-1}$ ($p \leq 0.001$), respectively. The ΔnuoN mutant reached a final OD600 that was 4% ($p = 0.17$) less than WT and ΔnqrF1 reached a final OD600 that was 12% ($p \leq 0.01$) less than WT, potentially reflecting a reduced efficiency of the overall electron transport chain.

NADH dehydrogenase mutants exhibit reduced acid tolerance

To exacerbate the effects of the deletions, we cultured the mutant strains in acidic medium to cause an additional burden on membrane potential. We compared growth in minimal medium at pH 7.2 or 6.2 with 10 mM NAG or 20 mM D,L-lactate as the carbon source (Figure 6). Indeed, lower pH increased the defects of both mutant strains when grown with NAG. At lower pH, the ΔnuoN strain exhibited a stronger defect than the ΔnqrF1 strain. When grown with D,L-lactate, mutants grew similarly at pH 7.2 or pH 6.2.
indicating that acid stress alone was not a strong enough stressor on membrane potential to cause changes in growth with a substrate producing minimal NADH and relying on quinone-linked dehydrogenases.

A ΔnuoNΔnqrF1 double mutant is incapable of aerobic growth in minimal media

Because both ΔnuoN and ΔnqrF1 strains exhibited only minor differences compared to WT, we created a ΔnuoNΔnqrF1 double knockout strain to determine whether these complexes have overlapping function. In contrast to the single mutants, this double mutant is incapable of aerobic growth in minimal media supplemented with either 10 mM NAG or 20 mM d,l-lactate in 24-well plates (Figure 7). We also attempted to grow this strain in the same media in flasks and observed no change in OD600 over time (data not shown). Even in LB medium, this strain exhibited severely reduced growth compared with WT (Figure S2). Because the ΔnuoNΔnqrF1 strain was unable to grow in minimal media, we did not conduct additional analyses on its phenotype. We complemented the double mutant strain with either nuoN or nqrF1 expressed in trans from a multi-copy plasmid. We observed enhanced growth rates in LB for both complemented strains compared with ΔnuoNΔnqrF1 carrying a plasmid with GFP (Figure S3). This indicates that the growth defect observed for the double mutant was due to the absence of these genes, rather than an off-target effect.

Discussion

Implications of growth defects in NADH dehydrogenase mutant strains

Growth deficiencies in the single and double knockout strains show that both Nuo and Nqr1 are important for aerobic growth and metabolism in S. oneidensis MR-1. The subtle phenotypes of the single mutant strains indicate that the two complexes have significant functional overlap, and that it is not necessary for both to function under the tested conditions. However, the severe defect of the double mutant strain indicates that Nqr2 and Ndh are unable to compensate for the combined loss of Nuo and Nqr1, indicating that at least one is necessary. We hypothesize that Ndh cannot compensate because it does not generate PMF or SMF, and that Nqr2 cannot compensate because it is not significantly expressed under aerobic...
conditions. Our data suggests that Nqr1 plays a greater role than Nuo under the tested conditions due to the more significant phenotypes of the ΔnqrF1 strain. This is unsurprising, given the distribution of each of these in the genus, as discussed in the introduction. However, Nuo may play a more important role in managing acid stress, considering that at pH 6.2 a greater growth defect was observed for ΔnuoN than for ΔnqrF1. These data suggest that although the two NADH dehydrogenases are likely used concurrently during aerobic respiration, they also have distinct roles.

Changes in metabolism suggest inhibition of the TCA cycle by increased intracellular NADH

We observed that carbon sources that must be processed by the TCA cycle (and therefore, generate NADH) exacerbate the growth defect in the single mutant strains. Metabolism of both NAG and acetate rely primarily on NADH as the electron carrier to feed the electron transport chain, while lactate may be partially oxidized by quinone-linked dehydrogenases that bypass NADH. We observed that growth with lactate was much less sensitive to the NADH dehydrogenase deletions than growth with the other substrates. It is somewhat surprising that growth defects were stronger with acetate than with NAG, although this may be explained by the possibility to generate formate through pyruvate formate lyase during NAG (or lactate) metabolism, but not during acetate metabolism. Acetate metabolism may be further hindered by allosteric reduction of activity of TCA cycle enzymes by high levels of NADH in the mutant strains. Although we have no direct measurements of intracellular NADH levels, accumulation of extracellular acetate during growth on NAG and D,L-lactate suggests that insufficient TCA cycle activity in the mutants forces acetate excretion. In the case of NAG, the acetate may be produced by either initial removal of the acetyl group or by production of acetyl-coA after glycolysis, but either way, the acetate is being excreted rather than being processed.

Roles of sodium ion vs proton energetics in S. oneidensis MR-1

We observed that Nuo and Nqr1 have overlapping function but did not elucidate how electron flux is partitioned between them when both are present. Analysis of the Shewanella genus provides some
evidence that more flux would be directed to Nqr1, which would align well with our growth observations.

While most Shewanella isolates originate from marine environments, S. oneidensis MR-1 was isolated from a freshwater lake. This habitat difference seems to be reflected in Shewanella energetics (33).

Shewanella spp. generally utilize only sodium-ion based energetics, which can be advantageous in a marine environment, but S. oneidensis MR-1 has gained complexes for proton-based energetics through horizontal gene transfer. For example, all sequenced genomes in the Shewanella genus encode sodium ion-dependent Nqr NADH dehydrogenases, but S. oneidensis MR-1 is one of only a few strains that encode the proton-dependent NADH dehydrogenase, Nuo (19, 34). Similarly, S. oneidensis MR-1 is the only strain in the genus with both SMF- and PMF-driven flagellar machinery, while the rest of the genus only has the SMF-driven system (20). Previous studies have not observed strong defects for knocking out PMF-dependent systems (35, 36) and our results reveal slightly stronger defects for Nqr1 mutants than Nuo mutants. This suggests that sodium remains an important coupling ion in S. oneidensis MR-1 despite its acquisition of PMF-dependent machinery. The potential benefits of maintaining both systems in the S. oneidensis MR-1 genome remain unclear.

The co-utilization of proton- and sodium-dependent energetics in S. oneidensis MR-1 raises the question of whether SMF and PMF could be conserved separately for different cellular functions. While the ΔΨ component is shared between PMF and SMF, the ΔpH and Δ[Na+] components can vary and thereby influence the relative activity of PMF- versus SMF-dependent processes. S. oneidensis MR-1 preferentially utilizes its sodium-ion dependent stator, but its ATP synthase does not contain the necessary residues for sodium ion transport, indicating that it is proton-dependent (23, 24). This arrangement could allow S. oneidensis MR-1 to favor either motility or ATP synthesis by upregulating Nqr1 or Nuo, respectively. Antiporters may also be utilized to interconvert between ΔpH and Δ[Na+] and thereby allow adaptive utilization of ion gradients for different functions (25, 37). Further study is needed to determine whether S. oneidensis MR-1 utilizes its coupling ion flexibility to conserve energy for different purposes.
Implications of Na⁺ dependent energetics for bioelectrochemical technologies

The ability to use a sodium-based respiratory system may have wide-ranging impacts on the physiology of *S. oneidensis* MR-1 in bioelectrochemical systems, particularly because local pH extremes near electrodes can be a major limiting factor (38). Previous analysis of biocathode processes has suggested that sodium ion-based respiration may be advantageous for organisms on a biocathode because of high local pH at the electrode (38). The localized alkaline pH at the biocathode can lead to loss of PMF in the biofilm and hinder proton-dependent respiration (39). Therefore, *S. oneidensis* MR-1 may represent a promising chassis for engineering cathodic bioelectrochemical technologies, such as microbial electrosynthesis. Understanding the interactions between pH and the NADH dehydrogenases at the electrode would also allow us to better explore the genetic optimization of organisms in bioelectrochemical systems in general.

Perspectives for future work

To better understand the specific function of each dehydrogenase, it is necessary to generate triple knockout strains, leaving only one functional NADH dehydrogenase in the genome, wherever possible. Membrane preparations from such strains could be used to confirm activity of each of these dehydrogenases through biochemical assays. If these strains are nonviable, a recently developed CRISPR interference system could be utilized to ‘knock down’ expression of the NADH dehydrogenases (42). This study focused on Nqr1 and Nuo mutants because NqrF2 and Ndh mutants showed no growth defects in oxic conditions; however, we hypothesize that NqrF2 and Ndh mutants will exhibit growth defects under anoxic conditions. Future study of all four mutant strains under anoxic conditions would also provide the opportunity to explore a wider range of thermodynamic constraints on metabolism, which may influence respiratory efficiency.

Conclusion
Under the laboratory conditions tested, *S. oneidensis* MR-1 utilized both Nqr1 and Nuo to oxidize NADH and conserve energy during aerobic growth. While single mutants lacking the activity of either complex grew only slightly slower than WT, a double-knockout lacking both was completely incapable of growth in minimal media. Although two additional NADH dehydrogenases are encoded in the genome, either Nuo or Nqr1 was required for aerobic growth in minimal media. Changes in accumulation of acetate suggest that when either of these complexes is absent, intracellular NADH levels increase, and potentially inhibit TCA cycle activity. We suggest that co-utilization of Nuo and Nqr results in adaptive metabolic redundancy and may represent a mechanism by which *S. oneidensis* MR-1 could conserve energy for different purposes, such as motility (preferentially sodium ion-dependent) and ATP generation (likely proton-dependent).

**Materials and Methods**

**Analysis of a whole-genome fitness profiling dataset**

Fitness data for *S. oneidensis* MR-1 transposon mutants was downloaded as a supplementary information file for the whole-genome fitness profiling study by Deutschbauer et al. (31). Fitness values were averaged across each NADH dehydrogenase operon and across groups of conditions. Values for libraries grown in minimal medium were plotted.

**In-frame deletion of loci from the *S. oneidensis* MR-1 genome**

Deletions of target genes were made using the pDS3.0 non-replicative vector as previously described and confirmed by PCR (40). Fusion products were made via PCR, generating tagged complementary sequence fragments that were subsequently linked following the cross-over PCR protocol. Six primers were designed and used for each strain, with the unique tag sequences added to the 5i and 3i primers (Table 2). Fusion products were inserted into the pDS3.0 vector with T4 ligase and transformed into chemically competent *E. coli* WM3064 cells via heat shock. The plasmids were transferred to *S. oneidensis* MR-1 via a conjugation protocol similar to Webster et al. (11). Primary conjugants were screened for gentamycin.
resistance and insertion into the genome was confirmed via two PCR reactions: one reaction with FO and 3o primers; and another with 5o and RO primers. Primary integrants were grown for 8 hours in LB without NaCl, then plated on LB without NaCl and with 10% sucrose. Individual colonies were screened via PCR using FO and RO flanking primers (Table 2) to identify deletion mutants. To acquire the double mutant strain, it was necessary for the resolution step of the protocol to be extended from 18 hours to 22 hours. At 18 hours, the cells still maintained antibiotic resistance indicating that the plasmid had not yet resolved out of the genome of most cells.

Growth conditions

All strains were pre-cultured in LB medium (Miller, Accumedia) for 16 hours. Pre-cultures were washed with M5 medium and standardized to OD$_{600}$=1.0 prior to use. All growth experiments utilized the following M5 minimal medium recipe: 1.29 mM K$_2$HPO$_4$, 1.65 mM KH$_2$PO$_4$, 7.87 mM NaCl, 1.70 mM NH$_4$SO$_4$, 475 μM MgSO$_4$·7H$_2$O, 10 mM HEPES, 0.01% (w/v) casamino acids, 1X Wolfe’s mineral solution (Al was not included), and 1X Wolfe’s vitamin solution (riboflavin was not included), pH adjusted to 7.2 with 1 M NaOH. Either NAG or sodium D,L-lactate was added to a final concentration of 10 mM or 20 mM, respectively, unless noted otherwise. We conducted an additional experiment to ensure that differences in sodium concentration between NAG and D,L-lactate did not cause significant changes in growth (Figure S4). We did not observe any significant difference, therefore extra sodium was not routinely added to medium with NAG.

High-throughput growth experiments were performed in clear 24-well culture plates (Sigma, SIAL0526) using 1 mL M5 medium, with four replicates per strain. Wells were inoculated with 10 μL of standardized pre-culture (washed in M5 and diluted to OD$_{600}$=1) and incubated in a Synergy HTX plate reader (BioTek Instruments, Winooski, VT) at 30°C with maximal shaking amplitude and minimal shaking speed. OD$_{600}$ was recorded by the instrument at 15-minute intervals. High-throughput growth pH experiments were performed in 24-well plates as described above with the following modifications: M5 was prepared with the pH adjusted to 6.2 with 1 M HCl prior to autoclaving, 24-well plates were prepared...
with triplicates for each strain in each condition: M5 pH 7.2 and M5 pH 6.2.

Flask growth experiments were performed in 250-mL Erlenmeyer flasks using 50 mL of M5 media supplemented with either 20 mM D,L-lactate or 10 mM NAG. Flasks were inoculated with 50 µL of standardized pre-culture and incubated in a floor shaker (New Brunswick Scientific, 12500) at 30°C and shaking at 275 rpm. Cultures were grown in triplicate for 40 hours. Growth was monitored by removing 1 mL every two hours starting four hours post inoculation and measuring the OD_{600}. Samples were stored at -20°C prior to preparation for HPLC analysis.

**HPLC analysis**

HPLC analysis was performed on a Shimadzu 20A HPLC, using an Aminex HPX-87H column with a Micro-guard Cation H⁺ guard column (BioRad, Hercules, CA) at 55°C. Samples were analyzed with a 0.6 mL/min flow rate, in 5 mM sulfuric acid with a 30-minute run time. Eluent was prepared by diluting a 50% HPLC-grade sulfuric acid solution (Fluka) in Milli-Q water and then degassing the solution at 37°C for 3-5 days before use. Compounds of interest were detected by refractive index (RID-20A). Samples were prepared by centrifuging 1 mL samples taken from flask growth for 10 minutes at 13,000 x g in a microcentrifuge (Minispin Plus, Eppendorf) to remove cells. The supernatant was removed and transferred to a 2.0-mL glass HPLC vial. Standards were prepared at concentrations of 1, 2, 5, 10, and 20 mM for D,L-lactate, NAG, and sodium acetate. Samples were maintained at 10°C by an auto-sampler throughout analysis.

**Complementation**

The double mutant strain was complemented using an IPTG-inducible plasmid, pRL814 (a generous gift from Dr. Robert Landick, University of Wisconsin, Madison). pRL814 was isolated from *E. coli* using an E.Z.N.A plasmid DNA kit (Omega Bio-Tek). Prepared plasmid DNA was linearized using NdeI and HindIII (New England Biolabs). *S. oneidensis* MR-1 Genomic DNA was isolated using the UltraClean Microbial DNA isolation kit (MO Bio, Carlsbad, CA). Primers used to amplify *nuoN* or *nqrF1* from *S.
*S. oneidensis* MR-1 genomic DNA were generated using the NEBuilder tool (New England Biolabs, Ipswich, MA). Linearized pRL814 and *nuoN* or *nqrF1* were assembled using NEBuilder High Fidelity DNA assembly kit (New England Biolabs, Ipswich, MA) using the standard protocol (43). Following assembly, *E. coli* WM3064 chemically competent cells were transformed with either pRL814-*nuoN* or pRL814-*nqrF1*. WM3064 strains were then used in conjugation with *S. oneidensis* MR-1 into the Δ*nuoN*Δ*nqrF1* strain. In parallel, WM3064 strains bearing unmodified pRL814 (which expresses GFP), were used in conjugation with wild type MR-1 and Δ*nuoN*Δ*nqrF1*. The control and complemented strains were grown in LB medium containing 100 μM IPTG and 50 μg/mL spectinomycin. Growth experiments were conducted in 24-well plates, with a 1 mL culture volume, in LB medium containing 100 μM IPTG and 50 μg/mL spectinomycin. Wells were inoculated with 10 μL of standardized pre-culture (washed in LB + 100 μM IPTG + 50 μg/mL spectinomycin and diluted to OD₆₀₀=1) and incubated in a Synergy HTX plate reader (BioTek Instruments, Winooski, VT) at 30°C with maximal shaking amplitude and minimal shaking speed. OD₆₀₀ was recorded by the instrument at 15-minute intervals.

**Data analysis**

Analysis of growth and HPLC data was performed using Rstudio (44) using the following packages: ggplot2 (45), reshape2 (46), dplyr (47), and TTR (48). Analysis of growth rates from flask growth experiments were performed using R package ‘growthcurver’ using default values with background correction set to “min” (49).

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**Figures**
Figure 1. Fitness of NADH dehydrogenase mutants under several growth conditions: D,L-lactate/oxic, D,L-lactate/anoxic, NAG/oxic, and NAG/anoxic. Values are averaged across loci in each operon and across multiple growth conditions that fit the descriptions above. Error bars represent standard deviation. Fitness is relative to aerobic growth in LB. All data were generated by Deutschbauer et al (43).

Figure 2. NAG and lactate metabolism in Shewanella oneidensis under aerobic conditions.
**Figure 3.** (A) Analysis of WT, ΔnuoN, and ΔnqrF1 grown in 50 mL M5 minimal medium with 10 mM NAG in a 250-mL flask. (B) NAG concentration in culture supernatant, and (C) acetate concentration in culture supernatant.

**Figure 4.** (A) Analysis of WT, ΔnuoN, and ΔnqrF1 grown in 50 mL M5 minimal medium with 20 mM D,L-lactate in a 250-mL flask. (B) D,L-lactate concentration in culture supernatant, (C) acetate concentration in culture supernatant.
Figure 5. Growth of WT, ΔnuoN, and ΔnqrF1 in 1 mL of M5 medium containing 10 mM sodium acetate in a 24-well plate.

Figure 6. Growth of WT, ΔnuoN, and ΔnqrF1 in 1 mL of M5 media containing (A) 10 mM NAG or (B) 20 mM D,L-lactate at a pH of 6.2 or 7.2 in 24-well plates.
Figure 7. Growth of WT, ΔnuoN, ΔnqrF1, and ΔnuoNΔnqrF1 in 1 mL of M5 medium containing (A) 10 mM NAG or (B) 20 mM D,L-lactate in 24-well plates.

Table 1. Growth rate analysis of Shewanella oneidensis NADH dehydrogenase mutants in 50 mL M5 minimal medium with 10 mM NAG, 20 mM D,L-lactate in 250 mL flasks and in 1 mL M5 with 10mM acetate in a 24-well plate. Growth rates were calculated using the R package ‘growthcurver,’ which fits the growth curve data to the best-fit logistic curve (50).

| Carbon source | S. oneidensis | Growth rate (hr⁻¹) | Difference from WT (%) | p-values |
|---------------|---------------|---------------------|------------------------|----------|
| NAG           | WT            | 0.93 ± 0.05         | n/a                    | n/a      |
|               | ΔnuoN         | 0.76 ± 0.06         | 17.7                   | 0.020    |
|               | ΔnqrF1        | 0.81 ± 0.04         | 12.9                   | 0.027    |
| D,L-lactate   | WT            | 0.45 ± 0.05         | n/a                    | n/a      |
|               | ΔnuoN         | 0.41 ± 0.04         | 9.1                    | 0.33     |
|               | ΔnqrF1        | 0.43 ± 0.05         | 4.8                    | 0.60     |
| Acetate       | WT            | 0.55 ± 0.04         | n/a                    | n/a      |
|               | ΔnuoN         | 0.36 ± 0.02         | 35.0                   | 0.0018   |
|               | ΔnqrF1        | 0.32 ± 0.01         | 42.8                   | 0.0005   |
**Table 2.** List of primers used for generation of in-frame deletion mutants.

| Gene | Primer | Sequence                                      |
|------|--------|-----------------------------------------------|
| nuoN | FO     | CTCTCAATAGAGCAGCTC                         |
|      | RO     | ACCACGACATCCTCCACATG                      |
|      | 5O     | GCTCAATATGATTGCCGGGCT                     |
|      | 5i     | GGGATGAACACACCATGCTGCTGTTCAGTAACGC       |
|      | 3O     | ATCTACTCGGAGACCAAGTG                      |
|      | 3i     | TGACATGGTGTGTTCTATCCCCGGAGAAAGTGTAGGCG   |
|      |        |                                               |
|      | FO     | CTGGCCTCTCTCTCTGCTAT                     |
|      | RO     | GCTTATGCTGATCAATTCTCGG                    |
|      | 5O     | AGGGATGCGGTAGTGTTAGTG                     |
|      | 5i     | CACAGAATCAGCCTGCTGATTCCCTAATCCAGTGACCAGC |
|      | 3O     | CGCTTATAGCTTCCAAAAACCCCA                 |
|      | 3i     | GAATCAGCGGTATCTGCTGCTGTAATTAGCCCGG      |
|      |        |                                               |
|      | FO     | TACATATCTTCAACATGTTGATTAATA               |
|      | RO     | CTGAATGAAAATTAATAATTGAAGGG                 |
|      | 5O     | ATTTTCTGTTGTAGTGTTAGTTG                   |
|      | 5i     | ACCCATGACCCTAAAAATAGAAATAACACCCCTAAAAACTCAT |
|      | 3O     | CTAAAACCCGCTTACC                          |
|      | 3i     | TCAATTCTGAGTGGTGCTGCTGCTGTAAGAGACAGAGCT  |
|      |        |                                               |
|      | FO     | CGCTGTCGTTCTCTCTGCTGTTGTG                 |
|      | RO     | GTGCGGCTAGCTCGTTTTCG                     |
|      | 5O     | GTGCTAATGACCTTGAGCCGTGCC                 |
|      | 5i     | GGCTTGAGAGATCCTAACACCTCATCCGATAACC      |
|      | 3O     | CAATTTGATGCGCTTGATGAGAGTC                |
|      | 3i     | GTAGTGCGATCTCTCAAGCCCGATTAGGTTGTAAGT    |

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