Roles of the Sodium-Translocating NADH:Quinone Oxidoreductase (Na⁺-NQR) on *Vibrio cholerae* Metabolism, Motility and Osmotic Stress Resistance

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**Abstract**

The Na⁺ translocating NADH:quinone oxidoreductase (Na⁺-NQR) is a unique respiratory enzyme catalyzing the electron transfer from NADH to quinone coupled with the translocation of sodium ions across the membrane. Typically, *Vibrio* spp., including *Vibrio cholerae*, have this enzyme but lack the proton-pumping NADH:ubiquinone oxidoreductase (Complex I). Thus, Na⁺-NQR should significantly contribute to multiple aspects of *V. cholerae* physiology; however, no detailed characterization of this aspect has been reported so far. In this study, we broadly investigated the effects of loss of Na⁺-NQR on *V. cholerae* physiology by using Phenotype Microarray (Biolog), transcriptome and metabolomics analyses. We found that the *V. cholerae* ΔnqrA-F mutant showed multiple defects in metabolism detected by Phenotype Microarray. Transcriptome analysis revealed that the *V. cholerae* ΔnqrA-F mutant up-regulates 31 genes and down-regulates 55 genes in both early and mid-growth phases. The most up-regulated genes included the cadA and cadB genes, encoding a lysine decarboxylase and a lysine/cadaverine antiporter, respectively. Increased CadAB activity was further suggested by the metabolomics analysis. The down-regulated genes include sialic acid catabolism genes. Metabolomic analysis also suggested increased reductive pathway of TCA cycle and decreased purine metabolism in the *V. cholerae* ΔnqrA-F mutant. Lack of Na⁺-NQR did not affect any of the Na⁺ pumping-related phenotypes of *V. cholerae* suggesting that other secondary Na⁺ pump(s) can compensate for Na⁺ pumping activity of Na⁺-NQR. Overall, our study provides important insights into the contribution of Na⁺-NQR to *V. cholerae* physiology.

**Introduction**

Na⁺-translocating NADH:quinone oxidoreductases (Na⁺-NQR) are found in the respiratory chains of a number of marine and pathogenic bacteria [reviewed in [1,2]]. In organisms that express Na⁺-NQR, this enzyme is the gateway for electrons into the respiratory chain. Na⁺-NQR accepts reducing equivalents from NADH and donates them to the quinone pool [3,4]. The energy from this redox reaction is used to pump sodium ions from the inner to the outer side of the membrane, thereby building a sodium motive force (SMF).

Besides Na⁺-NQR, two more types of respiratory NADH-dehydrogenases have been reported, the proton-pumping NADH dehydrogenases (NDH-1 or Complex I) and NDH-2 [5]. Similar to Na⁺-NQR, NDH-1 utilizes energy from the redox reaction, but it pumps protons from the inner to the outer side of the membrane to build a proton motive force (PMF). Unlike Na⁺-NQR and NDH-1, NDH-2 has neither H⁺ nor Na⁺ efflux activities. A few pathogenic bacteria, such as *Yersinia pestis* and *Pseudomonas aeruginosa*, have all three types of NADH dehydrogenases, but most bacteria possess one or two of these NADH dehydrogenases [5,6].

Lack of the major NADH dehydrogenase activity usually significantly affect bacterial physiology. *Escherichia coli* has a single NDH-1 (encoded by *nuad-Y*) and NDH-2 (encoded by *ndh*), and it is known that NDH-2 is the major enzyme for aerobic growth, whereas the NDH-1 is essential for anaerobic fumarate and DMSO respiration [7]. Moreover, it is known that the *E. coli* strain lacking NDH-1 showed growth retardation and increased acetate secretion after the transition to stationary growth phase when grown in mixed amino acids media [8]. It was also shown that the
**Materials and Methods**

**Bacterial strains and growth conditions**

*V. cholerae O395N1*, *V. cholerae O395N1 ΔnqrA-F* [12] and *V. cholerae O395N1 ΔpomAB* [13] strains were used in this study. Bacterial strains were kept at −80°C in 20% glycerol stocks. Bacterial strains were grown in Luria-Bertani (LB) medium at 30°C or 37°C. Streptomycin was supplemented at 100 μg/ml.

**Phenotype microarray (PM) analyses**

Phenotype microarrays were performed using PM3 to PM10 MicroPlate (Biolog) at Biolog’s PM Services group. All plates used pyruvate as sole carbon sources.

**DNA microarray analyses**

Cells of *V. cholerae O395N1* and *V. cholerae O395N1 ΔnqrA-F* strains, grown in LB (pH 6.5) at 30°C for 4 hours and 8 hours, were treated with RNA Protect Bacteria Reagent (Qiagen). RNA was extracted using the QIAGEN RNeasy Mini Kit (Qiagen). RNA was concentrated using the QIAGEN MiniRNeasy Cleanup kit (Qiagen) and sent to the Center for Genome Research and Biocomputing at Oregon State University, Corvallis, OR, following protocols outlined in the NimbleGen array user’s guide, version 5 for cDNA synthesis and microarray analysis using NimbleGen Microarray plates and reagents. The microarray data are accessible at the Gene Expression Omnibus (accession number, GSE56387).

**Metabolite extraction**

*V. cholerae O395N1* and *V. cholerae O395N1 ΔnqrA-F* strains were grown in LB (pH 6.5) at 30°C for 2 hours and the supernatant was removed by centrifugation (6000 rpm, +4°C, 5 min). The pellet was resuspended in 600 μL of LC-MS-grade water in 1 mM HEPES and 1 mM EDTA (pH 7.2). Metabolites were extracted from bacterial cells by using the boiling water method as previously described [14].

**LC-MS/MS**

Mass spectrometry based metabolomic profiling was performed as previously described [15]. Briefly, liquid chromatography (LC) was performed on a Shimadzu Nexera xSelect UPLC and metabolites separated on an Inertsil phenyl-3 stationary phase (GL Sciences, 5 μM, 4.6 × 150 mm). Mass spectrometry was performed on an AB SCIEX Triple TOF 5600 quadrupole-time-of-flight mass spectrometer. MS/MS spectra were gathered on the fly by information dependent acquisition. Most metabolites were identified by mass, isotope distribution, MS/MS fragmentation, and when standards were available, retention time. In the absence of chemical standards, MS/MS spectra were compared to those in the METLIN online database.

To account for analytical and sample preparation variation, samples were normalized to total ion count. Central energy metabolites (metabolites of the tricarboxylic acid cycle, pentose phosphate pathway, and glycolysis and amino acids) were targeted post-data acquisition and in addition, untargeted statistical analysis (Student’s t-test p-value plotted against fold-change) revealed large (>10-fold) changes in cyclic AMP and cyclic GMP, and subsequently, related metabolites (purines and pyrimidines) were targeted post-data acquisition.

**Growth measurement**

Growth measurement assays were performed as previously described [16]. Briefly, overnight grown bacterial cells were inoculated into 200 μl liquid medium in 96-deep-well plates (Whatman) at an initial OD600 of 0.05 and grown at 37 °C for 18 h with vigorous aeration. LBB medium (non-cationic L broth) was supplemented with streptomycin and varying concentrations of ethylene glycol. The initial pH of the media was adjusted with HCl to 6.5 and buffered by the addition of 60 mM BTP. Growth was then measured as OD600 by scanning the plates on a Bio-Rad iMark microplate absorbance reader. All experiments were repeated at least three times in triplicate.

**Measurement of acetate excretion**

Acetate excretions from bacterial cells were measured as previously described [10].

**Motility assay**

Motility of *V. cholerae* strains was determined on soft agar plates buffered with 100 mM Bis/Tris (pH 6.5) containing 100 mM NaCl, 0.25% agar, 1% tryptone, 0.5% yeast extract and 50 μg ml⁻¹ streptomycin. The endogenous Na⁺ content of the medium was 11 mM as determined by atomic absorption spectroscopy [17]. If indicated, D,L-lactate (33 mM final concentration) was added from a stock solution adjusted to pH 6.5 with KOH. To inoculate the swarming plates, overnight cultures of the ΔnqrC-F mutant strain, its parent strain or the non-motile *V. cholerae O395-N1 ΔpomAB* [13] were diluted in LB medium to a starting OD600 of 0.01. Cells were grown until OD600 of 0.5–0.6 was reached and washed in fresh LB medium. Cell suspensions were adjusted to OD600 of 0.5 with LB, and aliquots of 1.5 μL were spotted on dried soft agar plates. The diameters of swarming diameters were determined after 16 h at 37°C. Mean values and standard error from 16 experiments are presented. P values were calculated using Student’s t-test.

**Results and Discussion**

The ΔnqrA-F mutant strain showed multiple defects in metabolism detected by the phenotype microarray

We had previously performed a Phenotype Microarray (Biolog) analysis to address how a ΔnqrA-F mutation affects *V. cholerae* metabolism and changes in metabolic profiles were determined for the *V. cholerae* parent strain and the ΔnqrA-F mutant strain [10]. We had found that the ΔnqrC-F mutant has defects in the utilization of several TCA cycle intermediates (fumaric acid, succinic acid and...
L-malic acid) and many carbon sources that are metabolized into TCA cycle intermediates (L-aspartic acid, L-histidine, L-glutamine and L-glutamic acid). However, because succinic acid was used as the carbon source in the PM3-PM8 plates (nitrogen source, phosphorus source, sulfur sources and nutrient supplements testing plates), the systematic metabolic defects observed in the $D_{nqrA-F}$ mutant and effects of $D_{nqrA-F}$ mutation on these metabolisms were still uncertain. Our previous Phenotype Microarray results indicated that the $D_{nqrA-F}$ mutant utilizes pyruvate as the carbon source similar to the parent strain. Consistent with this, the $D_{nqrA-F}$ mutant grew similar to the parent strain when grown in M9 minimal media supplemented with pyruvate as the sole carbon source (data not shown). Thus, we tested the PM3-PM8 again using pyruvate as a sole carbon source. The results of the phenotype microarray indicated that the $V. cholerae$ O395N1 $D_{nqrA-F}$ mutant strain still showed multiple defects in nitrogen, phosphate and sulfur utilization (Table S1). Using PM 9 and PM 10 plates, we also tested the effects of osmolytes and pH and found that the $D_{nqrA-F}$ mutant is sensitive to sodium chloride, sodium sulfate, ethylene glycol, and urea (Table S1). These data again confirmed that lack of Na$^+$-NQR broadly affects $V. cholerae$ metabolism.

**Transcriptome analyses**

We next performed DNA microarray analyses to investigate how the lack of Na$^+$-NQR broadly affects gene expression pattern in $V. cholerae$. We had previously found that the $D_{nqrA-F}$ mutant increased virulence gene expressions, including toxT, ctxB and tcpA, but only at the early phase of the logarithmic growth [18]. This finding suggested that the lack of Na$^+$-NQR affected $V. cholerae$ gene expressions differently at different phases of growth. Because we aimed to understand the overall changes of gene expression pattern in the $D_{nqrA-F}$ mutant strain, we performed microarray analyses using RNA prepared from bacterial cultures both at the early and late phases of the logarithmic growth. At the early logarithmic growth, 612 genes were up-regulated and 660 genes were down-regulated in the $D_{nqrA-F}$ mutant compared to the parent strain, whereas at the late logarithmic growth phase, 119 genes were up-regulated and 264 genes were down-regulated in the $D_{nqrA-F}$ mutant (data not shown). Consistent with our previous

| VC number | Functions | Fold change (early) | Fold change (mid) |
|-----------|-----------|---------------------|-------------------|
| VC0280    | lysine/cadaverine antiporter, cadB | 17.836 up | 8.313 up |
| VC0281    | lysine decarboxylase, cadA | 26.554 up | 4.988 up |
| VC0479    | hypothetical protein | 2.075 up | 1.516 up |
| VC0615    | endoglucanase-related protein | 1.709 up | 1.568 up |
| VC0620    | peptide ABC transporter, periplasmic peptide-binding protein | 1.686 up | 2.131 up |
| VC0786    | D-amino acid dehydrogenase small subunit | 2.512 up | 1.881 up |
| VC1203    | urocate hydratase | 3.598 up | 2.000 up |
| VC1204    | formimidoylglutamase | 3.152 up | 2.211 up |
| VC1205    | imidazolonepropionase | 2.674 up | 2.062 up |
| VC1480    | hypothetical protein | 1.677 up | 1.811 up |
| VC1481    | hypothetical protein | 1.654 up | 1.640 up |
| VC1627    | pH-dependent sodium/proton antiporter, nhaA | 2.199 up | 2.089 up |
| VC1689    | hypothetical protein | 2.292 up | 1.634 up |
| VC1752    | hypothetical protein | 1.611 up | 1.592 up |
| VC1827    | mannose-6-phosphate isomerase | 6.706 up | 1.662 up |
| VC1828    | hypothetical protein | 2.703 up | 1.629 up |
| VC2216    | hypothetical protein | 2.216 up | 1.527 up |
| VC2361    | autonomous glycyl radical cofactor GrcA | 2.197 up | 1.920 up |
| VC2556    | hypothetical protein | 1.572 up | 1.519 up |
| VC2699    | anaerobic C4-dicarboxylate transporter | 3.431 up | 1.509 up |
| VCA0029   | transcriptional regulator, putative | 3.633 up | 2.241 up |
| VCA0562   | hypothetical protein | 1.674 up | 1.585 up |
| VCA0702   | iron-containing alcohol dehydrogenase | 1.770 up | 1.841 up |
| VCA0732   | hypothetical protein | 2.365 up | 1.746 up |
| VCA0744   | glycerol kinase | 1.653 up | 2.770 up |
| VCA0773   | methyl-accepting chemotaxis protein | 2.453 up | 1.937 up |
| VCA0811   | N-acetylglucosamine-binding protein A | 7.632 up | 2.174 up |
| VCA0827   | pterin-4-alpha-carbinolamine dehydratase | 1.542 up | 2.797 up |
| VCA0948   | hypothetical protein | 2.456 up | 1.554 up |
| VCA1045   | PTS system, mannitol-specific IABC component | 1.732 up | 2.479 up |
| VCA1046   | mannitol-1-phosphate 5-dehydrogenase | 1.997 up | 1.712 up |

Table 1. Genes up-regulated in the $D_{nqrA-F}$ mutant based on microarray analysis.
| VC number | Functions | Fold change (early) | Fold change (mid) |
|-----------|-----------|---------------------|-------------------|
| VC0022    | hypothetical protein | 1.657 down | 1.644 down |
| VC0061    | thiamine biosynthesis protein ThiC | 1.916 down | 1.739 down |
| VC0062    | thiamine-phosphate pyrophosphorylase | 1.935 down | 1.724 down |
| VC0063    | thiF protein | 1.730 down | 1.864 down |
| VC0302    | putative 3-phenylpropionic acid transporter | 2.403 down | 1.821 down |
| VC0730    | copper homeostasis protein | 1.675 down | 1.647 down |
| VC0734    | malate synthase | 2.689 down | 1.789 down |
| VC0751    | co-chaperone HscB | 1.651 down | 1.828 down |
| VC0754    | hypothetical protein | 1.899 down | 1.755 down |
| VC0766    | exodeoxyribonuclease VII large subunit | 1.792 down | 2.076 down |
| VC0769    | chitinase, putative | 2.752 down | 1.532 down |
| VC0916    | phosphotyrosine protein phosphatase | 3.258 down | 1.622 down |
| VC0917    | UDP-N-acetylglucosamine 2-epimerase | 2.858 down | 2.191 down |
| VC1070    | phosphatase, putative | 1.752 down | 1.591 down |
| VC1124    | hypothetical protein | 1.561 down | 1.519 down |
| VC1267    | hypothetical protein | 1.761 down | 1.515 down |
| VC1312    | alanine racemase | 1.625 down | 1.767 down |
| VC1454    | RsaA1 protein | 3.940 down | 1.617 down |
| VC1461    | colonization factor | 2.246 down | 2.188 down |
| VC1777    | sialic acid-specific TRAP transporter, SiaP | 2.354 down | 1.992 down |
| VC1778    | sialic acid-specific TRAP transporter, SiaQ | 3.571 down | 1.923 down |
| VC1779    | sialic acid-specific TRAP transporter, SiaM | 3.150 down | 2.187 down |
| VC1782    | N-acetylmannosamine kinase | 7.239 down | 1.986 down |
| VC1783    | N-acetylglucosamine-6-phosphate deacetylase | 5.481 down | 1.767 down |
| VC1784    | neuraminidase | 2.475 down | 2.646 down |
| VC1927    | C4-dicarboxylate transport protein | 1.745 down | 1.763 down |
| VC1928    | C4-dicarboxylate transport protein DctQ, putative | 1.970 down | 1.947 down |
| VC1929    | C4-dicarboxylate-binding periplasmic protein | 2.449 down | 2.796 down |
| VC2037    | Na+/H+ antiporter, nhaC-1 | 1.680 down | 1.599 down |
| VC2127    | flagellar basal body-associated protein FlIL | 1.885 down | 1.602 down |
| VC2128    | flagellar hook-length control protein FlIK, putative | 4.759 down | 1.826 down |
| VC2130    | flagellum-specific ATP synthase | 2.060 down | 1.915 down |
| VC2131    | flagellar assembly protein H | 1.807 down | 1.805 down |
| VC2132    | flagellar motor switch protein G | 1.519 down | 1.863 down |
| VC2133    | flagellar MS-ring protein | 1.654 down | 1.547 down |
| VC2136    | sensory box sensor histidine kinase | 1.682 down | 1.533 down |
| VC2140    | flagellar capping protein | 1.824 down | 1.562 down |
| VC2141    | flagellar protein FlgG | 1.914 down | 1.611 down |
| VC2187    | flagellin | 1.617 down | 1.554 down |
| VC2190    | flagellar hook-associated protein FlgL | 5.302 down | 1.728 down |
| VC2192    | peptidoglycan hydrolase | 5.239 down | 1.591 down |
| VC2195    | flagellar basal body rod protein FlgG | 4.720 down | 1.538 down |
| VC2197    | flagellar hook protein FlgE | 2.561 down | 1.514 down |
| VC2705    | sodium/solute symporter, putative | 4.691 down | 1.963 down |
| VCA0176   | methyl-accepting chemotaxis protein | 2.111 down | 1.667 down |
| VCA0186   | hypothetical protein | 3.613 down | 1.670 down |
| VCA0204   | ATP-dependent RNA helicase RhlE | 1.708 down | 1.677 down |
| VCA0699   | glucose-1-phosphate adenylyltransferase | 1.700 down | 1.620 down |
| VCA0700   | chitodextrinase | 4.589 down | 1.619 down |
| VCA0835   | hypothetical protein | 1.876 down | 1.611 down |
data, virulence genes, including toxT, tcpA-F and ctxB were up-regulated in the ΔnqrA-F mutant at the early logarithmic growth phase but not at the late logarithmic growth phase (data not shown). Among these changes, 31 genes were commonly up-regulated and 55 genes were commonly down-regulated in both growth phases (Table 1 and 2).

The cadBA genes showed the highest increases in gene expression levels in the ΔnqrA-F mutant in both growth phases (Table 1). The cadB gene encodes a lysine decarboxylase and cadA encodes a lysine/cadaverine antipporter. The expression of cadBA is regulated by a ToxR-type transcriptional regulator, CadC [19], and a LysR type transcriptional regulator, AphB [20]. However, expression of the other AphB regulated genes, such as tcpP and nhaB [20], were not affected by the ΔnqrA-F mutation. Thus, CadC might be responsible for the induced expression of cadAB in response to lack of Na⁺-NQR. CadC is known to induce cadAB expression in response to acidic pH [19] and we previously found that the V. cholerae ΔnqrA-F mutant showed increased acetate production and caused acidification of the external medium [10]. Thus, it is likely that cadAB expression was induced by the acidic pH that results when Na⁺-NQR function is impaired.

Several transporter genes including tripartite ATP-independent periplasmic (TRAP) transporters, siaPQM and dctMQP, were down-regulated in the ΔnqrA-F mutant strain compared to the parent strain (Table 2). It was recently reported that SiaPQM is a Na⁺-dependent sialic acid-specific TRAP transporter [21,22].

Consistent with this, multiple genes encoding enzymes in the sialic acid utilization pathway, which convert sialic acid to fructose 6-phosphate, and neuraminidase, which convert host cell surface polysialogangliosides to GM1 monoganglioside and release sialic acid [23], were also down-regulated in the ΔnqrA-F mutant (Table 2). Given that Na⁺-NQR and sialic acid catabolic pathways are essential for V. cholerae colonization in the small intestine of mice [11,24], such decreased expression of genes in the sialic acid utilization pathway might explain why the ΔnqrA-F mutant showed defects in colonization in the small intestine of mice. The dctMQP genes were recently shown to encode a C4-dicarboxylate-specific TRAP transporter and to be partly responsible for V. cholerae C4-dicarboxylates, succinate, malate and fumarate utilization [21]. Thus, it might be possible that the decreased utilization of succinate, malate and fumarate by the V. cholerae ΔnqrA-F mutant [10] was simply caused by the decreased uptakes of these C4-dicarboxylates. Further studies to better investigate the intriguing links between gene regulation events in response to loss of Na⁺-NQR and changes in V. cholerae carbon utilization are required to better understand these observations.

We also found that two methyl-accepting chemotaxis proteins (MCPs), encoded by VCA0176 and VCA0864, were down-regulated in the V. cholerae ΔnqrA-F mutant compared to the parent strain (Table 2). Interestingly, we found that a VCA0864 mutant strain of V. cholerae C6706 showed a hypermotile phenotype in minimal media supplemented with N-acetylglucosamine chemotaxis plate (unpublished data). Together with the fact that the sialic acid degradation pathway and transport system were down-regulated in the V. cholerae ΔnqrA-F mutant, these data suggest that the ΔnqrA-F mutant might be sialic acids starved.

The Class II, III and IV flagellar genes were systematically down regulated in the ΔnqrA-F mutant compared to the parent strain (Table 2). One might speculate that the decreased amount of regulatory and structural components of the polar flagellum might result in diminished motility of the ΔnqrA-F mutant strain. Indeed, the diameter of its swarming rings on soft agar plates (pH 6.5) reached only 80% of the diameter observed with the parent strain, but was clearly motile when compared with the non-motile ΔpomAB V. cholerae strain lacking essential components of the flagellar stator.

When lactate was added, both parent and mutant strain exhibited diminished swarming, and the improved motility of the parent strain was no longer apparent (p = 1, Fig.1). This indicates that depending on the external medium, the mutant strain suffers from a limitation in energy supply for flagellar rotation, resulting in diminished motility.

### Metabolomic analyses
To further understand how lack of Na⁺-NQR affects V. cholerae metabolism, we performed a mass spectrometry based metabolomic profiling. We found that 12 metabolites were significantly increased in the ΔnqrA-F mutant strain compared to the parent strain.
decreased and 3 metabolites were increased in the *V. cholerae* ΔnqrA-F mutant compared to the parent strain (Table 3).

We had previously suggested that TCA cycle activity is decreased in the *V. cholerae* ΔnqrA-F mutant [10]. Consistent with this, intracellular isocitrate levels were decreased in the ΔnqrA-F mutant (Table 3). However, we unexpectedly found that intracellular malate levels were significantly increased in the ΔnqrA-F mutant (Table 3). We also detected slightly increased levels of succinate in the ΔnqrA-F mutant (data not shown). Taken together, this may suggest that the reductive pathway of the TCA cycle is more active in the *V. cholerae* ΔnqrA-F mutant (Fig. 2).

We found that intracellular lysine levels were decreased, while cadaverine levels were increased in the *V. cholerae* ΔnqrA-F mutant compared to the parent strain (Table 3). Since CadA catalyzes the conversion of lysine to cadaverine, these changes could be caused by the increased *cadA* expression that we detected in the transcriptome analyses.

The acetate switch is broken in the *V. cholerae* ΔnqrA-F mutant

We had previously shown that the *V. cholerae* ΔnqrA-F mutant showed increased acetate production compared to the parent strain [10]. When grown in tryptone-based rich media, *E. coli* first produces acetate by using the PTA-ACK pathway but at a certain point in its growth phase, it shifts to utilize the excreted acetate by using the AMP forming acetyl-CoA synthetase (AMP-ACS). This transition from acetate production to acetate utilization is called the acetate switch [25]. To further examine acetate production in

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**Table 3.** Metabolites changed in the ΔnqrA-F mutant based on metabolomics analysis.

| Metabolite         | Fold change (nqr/WT) | p-value (t-test) |
|--------------------|----------------------|------------------|
| Adenine            | 0.737                | 0.0246           |
| Adenosine          | 0.248                | 0.0299           |
| AMP                | 1.105                | 0.6629           |
| Arginine           | 0.964                | 0.4638           |
| Asparagine         | 0.936                | 0.1727           |
| Cadaverine         | 2.400                | < 0.0001         |
| Cyclic AMP         | 0.095                | 0.0066           |
| Cyclic GMP         | 0.094                | 0.0035           |
| Deoxyribose        | 0.769                | 0.1178           |
| dGMP               | 0.260                | 0.0025           |
| Fructose-1,6-bisphosphate | 0.772 | 0.2946      |
| Glucose            | 0.795                | 0.1613           |
| Glutamate          | 0.875                | 0.0220           |
| GMP                | ~ 0.10              | N/A              |
| Guanine            | 0.772                | 0.0065           |
| Guanosine          | 0.758                | 0.0056           |
| Histidine          | 0.956                | 0.4699           |
| Hypoxanthine       | 1.411                | 0.0222           |
| Inosine            | 0.594                | 0.0003           |
| Iso/citrate        | 0.756                | 0.0027           |
| Isoleucine         | 0.927                | 0.1445           |
| Lactate            | 0.739                | 0.0024           |
| Lysine             | 0.596                | 0.0004           |
| Malate             | 1.809                | 0.0003           |
| Methionine         | 0.873                | 0.1405           |
| Phenylalanine      | 0.886                | 0.0961           |
| Phosphoglycerate   | 1.094                | 0.7458           |
| Proline            | 0.896                | 0.2215           |
| Ribose phosphate   | 0.821                | 0.1549           |
| Serine             | 0.899                | 0.1519           |
| Succinate          | 1.664                | 0.0934           |
| Threonine          | 0.898                | 0.0837           |
| Tryptophan         | 0.818                | 0.0599           |
| Tyrosine           | 0.866                | 0.1397           |
| Uracil             | 1.461                | 0.0010           |
| Valine             | 0.861                | 0.1073           |

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the *V. cholerae* \( \Delta nqrA-F \) mutant, we measured a time course of acetate excretion. Between 6 hr and 8 hr of growth in LB media, the *V. cholerae* parent strain started to reduce external acetate production, indicating that *V. cholerae* also has an acetate switch (Fig. 2). Interestingly, the *V. cholerae* \( \Delta nqrA-F \) mutant did not show this phenotype and kept excreting acetate until the end of growth (Fig. 3). This is consistent with the Phenotype Microarray data that showed that the *V. cholerae* \( \Delta nqrA-F \) mutant had a defect in acetate utilization (Table S1). The AMP-ACS protein is known to be regulated by protein acetylation with the acetylated form of AMP-ACS being inactive and these defects in acetate utilization in the \( \Delta nqrA-F \) mutant suggested that AMP-ACS might be acetylated.

The \( \Delta nqrA-F \) mutation does not affect osmotic resistance in *V. cholerae*

The results of the phenotype microarrays showed that the *V. cholerae* \( \Delta nqrA-F \) mutant strain was sensitive to osmotic stress including NaCl and ethylene glycol (Table S1). Thus, we performed growth assays in LBB-based medium (noncationic LB broth), containing increasing concentrations of NaCl and ethylene glycol. Consistent with the phenotype microarrays, the *V. cholerae* \( \Delta nqrA-F \) mutant showed growth defect compared with the parent strain but the growth defect was independent to the increasing concentrations of NaCl (Quinn et. al. submitted) or ethylene glycol (data not shown). To further determine whether the effect of Na\(^{+}\)-NQR on growth was primarily related to loss of quinone reduction or more general Na\(^{+}\) homeostasis, L-lactate was added to the growth media, which is expected to allow the L-lactate dehydrogenase to replenish the quinone pool directly. Interestingly, addition of L-lactate restored the growth of the *V. cholerae* \( \Delta nqrA-F \) mutant to the parent level even in the presence of high concentration of NaCl (Quinn et.al. submitted) or ethylene glycol (data not shown). These data indicated that lack of Na\(^{+}\)-NQR does not directly affect osmotic stress resistance in *V. cholerae*. Our transcriptome analyses detected the increased expression of a major Na\(^{+}/H^{+}\) antiporter gene, *nhaA* in the *V. cholerae* \( \Delta nqrA-F \) mutant (Table 1), suggesting that NhaA might complement the Na\(^{+}\) pumping activity of Na\(^{+}\)-NQR.

**Conclusions**

Lack of Na\(^{+}\)-NQR broadly affects *V. cholerae* physiology but mainly affects its central metabolism but not Na\(^{+}\) pumping-related phenotypes.

**Supporting Information**

Table S1 Phenotypes of the *V. cholerae* \( \Delta nqrA-F \) mutant based on Biolog phenotype microarrays. (XLSX)
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Author Contributions

Conceived and designed the experiments: YM SRF CCH. Performed the experiments: YM SRF JSK PH MJQ WJF. Analyzed the data: YM JSK JS JFS CCH. Wrote the paper: YM SRF JSK AMA JS CCH.

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