AN ABSTRACT OF THE THESIS OF

Sara R. Fassio for the degree of Honors Baccalaureate of Science in Microbiology and Honors Baccalaureate of Arts in Spanish presented on June 7, 2013. Title: Characterization of the Sodium Transporting NADH:Quinone Oxidoreductase (NQR) in *Vibrio cholerae*.

Abstract approved:

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Claudia C. Häse

Vibrio cholerae is the causative agent of cholera, a serious diarrheal disease in developing countries. *V. cholerae* has a unique redox driven respiration-linked sodium pump, Na^+ translocating NADH:quinone oxidoreductase (NQR). Several reports previously showed that NQR plays an important role in virulence, metabolism, and sodium homeostasis of *V. cholerae*. This study aims to better understand the mechanisms of the effect of NQR on *V. cholerae* virulence, metabolism, and physiology. Analysis of the *V. cholerae* Δ*nqr* mutant revealed that virulence gene expression is highly growth-phase dependent and virulence gene transcription is altered only at the early-log growth phase compared to the wild type. The Δ*nqr* mutant also showed a broad range of metabolic defects, as assessed by a Phenotype Microarray™ (Biolog), suggesting the importance of NQR in the energy metabolism of *V. cholerae*. Furthermore, we also concluded that the effects of NQR on *V. cholerae* virulence gene expression were linked to respiration rather than sodium motive force. Taken together, these results suggest that NQR has broad growth dependent effects on *V. cholerae* metabolism and the observed changes in metabolism are linked to the observed changes in virulence gene expression following the loss of NQR.

Key Words: *Vibrio cholerae*, cholera, Na^+- transporting NADH:quinone oxidoreductase (NQR), ToxT, cholera toxin (CT), metabolism, Biolog Phenotype Microarray™

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Characterization of the Sodium Transporting NADH:Quinone Oxidoreductase (NQR) in *Vibrio cholerae*

by

Sara R. Fassio

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I understand that my project will become part of the permanent collection of Oregon State University, University Honors College. My signature below authorizes release of my project to any reader upon request.

Sara R. Fassio, Author

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Contribution of Authors

Parts of this project are derived from peer-reviewed publications or manuscripts for which I am co-authored along with other collaborators. Yusuke Minato, Alan J. Wolfe, and Claudia C. Häse are co-authors on these publications. All authors contributed extensively to work presented in the publications. Y.M. and S.R.F. designed and performed experiments, Y.M., S.R.F., A.J.W., and C.C.H analyzed the data and C.C.H. supervised the project.

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This thesis is dedicated to Erin and Joe Fassio for their undying support and for making my college education possible.

Characterization of the Sodium Transporting NADH:Quinone Oxidoreductase (NQR) in *Vibrio cholerae*

Background

Cholera

Cholera is a waterborne disease resulting from an infection of *Vibrio cholerae*, causing severe diarrhea and electrolyte loss. Cholera is contracted through ingestion of contaminated water or food. The watery diarrhea is distinctive in its "rice water" appearance and can exceed 20 liters per day. If left untreated acute dehydration and death can occur, often within 10-18 hours of onset of symptoms (1). There are an estimated 3-5 million cholera cases a year resulting in 100,000-120,000 deaths (1). Deaths primarily occur in areas with poor sanitation and contaminated water supplies, making cholera a serious public health concern in developing countries. While approximately 75% of cholera cases are asymptomatic, *Vibrio cholerae* are shed in the feces and have the potential to enter the environment and contribute to further water contamination (1). Those with lowered immunity, such as the malnourished or immunocompromised HIV patients, are particularly at risk for contracting cholera and have an increased risk of death (1). The largest, most recent epidemic of cholera occurred in 2010 in Haiti after an earthquake devastated the country. Sewage from a UN peacekeeping camp is believed to have contaminated a tributary to the main river, which resulted in the spread of cholera throughout every region of the country and caused more than 3,500 deaths (2). Due to

major outbreaks in the recent past, cholera is now widely considered a re-emerging infectious disease.

The mainstay treatment of cholera is intravenous and oral rehydration therapy. In the absence of oral rehydration therapy, mortality can exceed 60% but is reduced to less than 1% when electrolytes and lost fluids are replaced (1). Antibiotics are often used alongside rehydration therapy, particularly if the patient is severely dehydrated and continues to pass voluminous amounts of stool. Local antibiotic resistance patterns must be carefully assessed when administrating antibiotics, especially during an epidemic. Effectiveness of antibiotics is highly variable but generally lessens the severity of symptoms by reducing the duration of diarrhea by 50-56%, volume of stool output by 8-92%, and the duration of positive stool culture by 26-83% (3-7). Daily treatment costs for a cholera infection is about $1.60 per patient. Oral rehydration salts cost approximately $0.20 per person (8) while 1 tablet of Doxycycline costs approximately $0.02 per 100mg tablet and Azithromycin $0.16 per 250mg tablet (9). Despite the low cost of treatment and the simplicity of administration mortality continues to be high due to inaccessibility to treatment and clean water.

There is currently no cholera vaccine that offers sufficient protection for all populations. However, clinical exposure to *V. cholerae* O1 offers significant protective immunity (10). A vaccine consisting of whole killed *V. cholerae* cells has been tested and used but it fails to protect children and has the potential to induce diarrhea (11). Another vaccine consisting of live bacteria with a *ctx* (cholera toxin) gene deletion has also been tested but fails to induce sufficient protection (11). Vaccination programs could greatly benefit those with risk of exposure to cholera, including health care workers in

developing countries. The use of vaccination and antibiotics should be coupled with much needed improvements to sanitation and accessibility to potable water.

Vibrio cholerae

V. cholerae is a Gram negative, facultative anaerobic, gammaproteobacterium with a comma shape and single polar flagellum characteristic of the *Vibrio* species. There are over 200 serotypes of *V. cholerae* based on the O-antigen but only the O1 and O139 toxigenic serotypes are associated with major cholera epidemics (12). Although *V. cholerae* is a human pathogen, its primary habitat is an aquatic environment and it can be found in both freshwater and saltwater. *V. cholerae* has the ability to utilize the chitinous exoskeletons of zooplankton as a sole nutrient source. Chitin also induces natural competence for transformation, which is a mode of horizontal gene transfer for *V. cholerae* (13).

V. cholerae has a diverse lifecycle from aquatic reservoir to human infection. Formation of biofilms on biological substances such as exoskeletons, insect egg masses, copepods, and phytoplankton aids in the persistence of the bacterium. Upon ingestion of a contaminated food or water source, *V. cholerae* colonizes in the small intestine of the host, multiplies, secretes cholera toxin, and is then shed from the host in watery diarrhea. The stool-shed bacteria are hyperinfectious and serve to amplify the outbreak through transmission to subsequent hosts (14).

There are two main virulence factors that *V. cholerae* uses to establish infection: cholera toxin (CT) and toxin-coregulated pilus (TCP). CT is a type AB toxin that binds to its receptor GM_1 gangliosides in the small intestine, acting as a potent enterotoxin

responsible for inducing most of the cholera symptoms. Upon uptake into the cells, CT induces elevated levels of cyclic adenosine monophosphate (cAMP), which ultimately results in electrolyte and water loss as manifested by the watery diarrhea (15). Thus, CT reverses the normal physiology of the small intestine and causes it to secret water rather than absorb it. CT is also important in the adhesion and dissemination of *V. cholerae*. TCP is a type IV pilus that is essential for the colonization of the epithelial mucosa. Mutations in the *tcpA* gene, which encodes for TCP, resulted in a marked decrease of cholera symptoms in human volunteers (16). CT and TCP are co-regulated by a complex cascade of factors. The genes that encode TCP, CT, and many other virulence determinants comprise a network of genes called the ToxT regulon, whose expression is modulated by a hierarchy of transcriptional regulators (17). These regulators include the transcription factors AphA and AphB, which positively regulate transcription of the two-component regulatory system *tcpPH*. The membrane-bound protein complex TcpPH works with the membrane-bound protein complex ToxRS to activate transcription of *toxT*, which encodes an AraC-type transcriptional factor (18). ToxT directly activates the genes that encode TCP and CT. Expression of *toxT* in *V. cholerae* is tightly regulated and occurs only under limited conditions, including at the early stage of the infection in the small intestine. External stimuli such as temperature, pH, osmolarity, amino acids, and bile alter the expression of *toxT* (19). During the early stages of infection, *V. cholerae* can adapt its metabolism based on available nutrients and express virulence genes such as *toxT*. Thus, understanding *V. cholerae* metabolism might be key to understanding virulence gene regulation in this organism.

NQR

V. cholerae contains a unique redox-driven sodium pump, the sodium transporting NADH:quinone oxidoreductase (NQR). NQR is a respiratory enzyme that catalyzes the oxidation of NADH to NAD^+ and donates electrons to the quinone pool, coupled with the translocation of sodium ions across the membrane (Figure 1) (20, 21). The energy from this redox reaction is used to pump sodium from the inner to the outer side of the membrane, building a sodium motive force (SMF), which is used for metabolic work. NQR is found in the respiratory chains of a number of marine and pathogenic bacteria, including *Haemophilus influenzae*, *Neisseria gonorrhoeae*, *Yersinia pestis* and *Pseudomonas aeruginosa* and acts as a gateway for electrons into the respiratory chain (22, 23). Genomic analysis of *V. cholerae* showed it does not contain an ortholog of Complex I in the electron transport chain (ETC), NADH:ubiquinone oxidoreductase (NUO), which often acts as the main respiratory NADH dehydrogenase in some bacteria, including *Escherichia coli* (24). This analysis suggests that NQR plays an important role in the energy metabolism of *V. cholerae*.

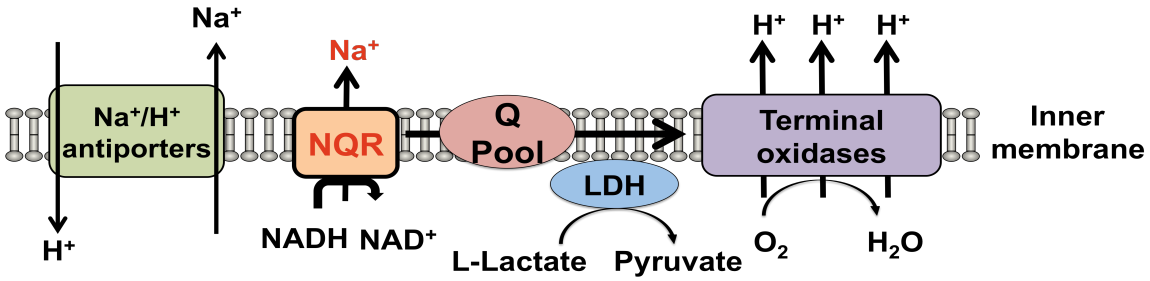


Figure 1. NQR in the aerobic system of *V. cholerae*. The electron transport system of *V. cholerae* possesses a redox driven sodium pump (NQR) instead of the NADH:ubiquinone oxidoreductase (NUO) found in *E. coli* and other bacteria. LDH: lactate dehydrogenase, Q: quinone

Häse *et al.* previously reported that mutational or chemical inactivation of NQR increased *toxT* expression in *V. cholerae* (25, 26). Since NQR is expected to generate a SMF, lack of functional NQR can be expected to reduce SMF-dependent flagella rotation. Since motility is correlated to virulence gene expression (27), we previously proposed that NQR might affect *toxT* expression via flagella rotation (28). Further characterization of this link, however, revealed that NQR-associated *toxT* induction is likely flagellum-independent because elevated *toxT* expression was observed in strains carrying a Δ*nqr* mutation in combination with motility defects (28). An earlier study also showed that this induction occurs independently of ToxR (25). However, the detailed mechanism by which NQR affects *toxT* transcription remains unknown.

Statement of purpose

Merrell *et al.* previously found that NQR plays an important role in the metabolism and sodium homeostasis of *V. cholerae* (29). That study, combined with the previous studies by Häse and Mekalanos (25, 26) that showed a link between NQR and virulence gene expression, have established the importance of NQR in *V. cholerae*. However, the detailed mechanisms of how NQR affects *toxT* expression, metabolism, and the physiology of the cell remain unknown. This study is an attempt characterize the broad affects of NQR on *V. cholerae* metabolism and physiology as well as clarify the mechanisms in which NQR influences virulence gene expression.

Methods

Bacterial strains and growth conditions

Bacterial strains *V. cholerae* O395N1, CA401, N16961, O395N1 Δ*nqr* and CA401 Δ*nqr* were used in this study. For Beta-galactosidase assays, CT-ELISAs, and acetate assays, bacterial strains were grown overnight in Luria-Bertani (LB) medium (Difco) at 37°C, washed, diluted to OD_{600} = 0.05 in unbuffered LB (initial pH 6.5) and then grown for 6 hr at 30°C in a roller drum. Media pH were adjusted by HCl or NaOH. For CT-ELISA, *V. cholerae* N16961 was grown in yeast extract peptone (YEP) under AKI conditions as previously described for the CT-ELISA (30, 31). Briefly, 10 μL of cell culture was inoculated into 10mL YEP and grown stationary at 37°C in test tubes for 4 hours. The cells were then shaken for 16 hours in 125mL flasks at 37°C.

As required, antibiotics were supplemented as follows: streptomycin at 100 μg/ml; ampicillin at 100 μg/ml; chloramphenicol at 1 μg/ml for *V. cholerae* and 10 μg/ml for *Escherichia coli*, and kanamycin at 50 μg/ml. Bacterial strains were kept at -80°C in 20% glycerol stocks.

Quantitative reverse transcription-polymerase chain reaction (qRT-PCR) analyses

V. cholerae O395N1 Δ*nqr* cells grown in unbuffered LB (pH 6.5) at 30°C in a roller drum were treated with RNA Protect Bacteria Reagent (Qiagen) after 2,4,6, and 8 hours. RNA was extracted using the QIAGEN RNeasy Mini Kit (Qiagen). Real-time qRT-PCR reactions were performed using the SuperScript® III Platinum® SYBR® Green One-Step

qRT-PCR Kit (Invitrogen) and ABI PRISM 7500 FAST Sequence Detection System (Applied Biosystems) at the OSU CGRB facility.

Cholera Toxin Enzyme-linked Immunosorbent Assay (CT-ELISA)

V. cholerae O395N1 wild type (WT) and Δ*nqr* and CA401 WT and Δ*nqr* cells were grown at 30°C in unbuffered LB (pH 6.5) for 6 hours in a roller drum. *V. cholerae* N16961 were grown at AKI conditions as described above. 1 mL samples of bacterial culture were taken after growth and centrifuged at 6,000x g for 10 min. The supernatants were then diluted 10 fold or 100 fold and plated onto GM1-ganglioside coated ELISA plates. Anti-cholera toxin antibody (Abcam) was used as the primary antibody, with goat anti-rabbit conjugated to horseradish peroxidase used as the secondary antibody (Bio-Rad) and the HRP substrate kit (BioRad) as the detecting reagent. Serial dilutions of the cholera toxin B subunit (Sigma) starting at 1 mg/ml were used to generate the standard curve.

Phenotype MicroArray™ (PM) analyses

Phenotype microarrays were performed using PM1 to PM10 MicroPlates™ (Biolog) at Biolog's PM Services group. PM1-PM2 plates contained carbon sources, while PM3-PM8 plates contained sulfur, phosphorus, and nitrogen sources and nutrient supplements. PM9 and PM10 plates tested for osmotic and pH sensitivity.

Microarray Analyses

Cells of *V. cholerae* O395N1 WT and Δ*nqr*, grown in unbuffered LB (pH 6.5) in a roller drum at 30°C until 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 RNA MiniElute Cleanup kit (Qiagen) and sent to the OSU CGRB Core Lab facility for cDNA synthesis and microarray analysis using NimbleGen Microarray plates and reagents.

Beta-Galactosidase Assays

Beta-Galactosidase activity was measured essentially as previously described (25). Briefly, 100 μL cell culture was mixed with 900 μL "Z buffer" (25), 40 μL chloroform, and 40 μL 1% SDS. The assay mixture was then mixed with 250 μL 1mg/mL ortho-Nitrophenyl-β-galactoside (ONPG) and incubated at 30°C for 15 minutes. The reaction was stopped using 500 μL 1M sodium carbonate and the mixture was measured at OD_{420}.

Measurement of acetate excretion

Bacterial culture was centrifuged at 13,000 rpm (4°C) for 2 min. Acetic acid levels in the cell-free supernatant were measured using an acetic acid enzymatic assay kit (R-Biopharm). This kit uses a colorimetric assay to measure the amounts of acetate in the supernatant and assay measurements were taken at OD_{340}.

Results and Discussion

Growth phase dependency of virulence expression in the Δ*nqr* mutant

As a continuation of our previous findings that NQR is linked to virulence gene expression (25), this project aimed to better understand the overarching affects of NQR on *V. cholerae* virulence gene expression, physiology, and metabolism. In order to assess the role of NQR, we used a *V. cholerae* O395N1 strain with a deletion of the *nqr* gene in its entirety (Δ*nqrA-F* gene subunits) (32). Since this mutant lacked the gene in question, we were able to compare the growth profiles and virulence gene expressions of the wild type (WT) strain with the Δ*nqr* mutant and therefore attribute any observed differences in the mutant strain to the lack of *nqr*.

We first determined the growth profiles of the WT and Δ*nqr* at different time points. The Δ*nqr* mutant showed similar growth rates as the WT strain at the early-log growth phase (0-4 hours), but transitioned to slower growth rates at the mid-log growth phase (4 hours) compared to the parent strain when grown in LB at 30°C in a roller drum (Fig. 2A). Since NQR is expected to serve as a Complex I ortholog in *V. cholerae* we predicted that it would share a similar growth profile to other Complex I mutants in different organisms. Indeed, a similar growth defect was observed with an *E. coli* Complex I (Δ*nuoB*) mutant when grown in tryptone broth (33).

Further analysis of virulence gene expression in the Δ*nqr* mutant was performed by assessing relative mRNA levels with qRT-PCR. Gene expression levels of *tcpP* (encodes TCP), *ctxB* (encodes CT subunit B), and *toxT* were approximately 50-fold higher in Δ*nqr* compared to the WT at the early-log growth phase (0-4 hours) but then

leveled out to WT levels after 4 hours (Fig. 2B). These findings were significant because they shed light on the importance of the growth phase in respect to virulence gene expression. Häse *et al.* had previously reported that lack of *nqr* increased *toxT* transcription (25). While we confirmed that overall *toxT* production is increased in Δ*nqr*, increased transcription is only observed at the early-log growth phase. This led us to conclude that the transcription of *toxT* and other virulence genes controlled by ToxT is temporary and growth phase dependent.

We next determined cholera toxin production by CT-ELISA. In agreement with the gene expression data, CT production of the Δ*nqr* mutant was higher than the WT strain at the early log growth phase (0-4 hours) (Fig. 2C). However, at the late-log growth phase (8 hours) the WT CT production surpassed the Δ*nqr* mutant's CT production by slightly more than 2-fold (Fig. 2D), even though *ctxB* transcriptional levels of the WT and Δ*nqr* were similar at the late-log phase (Fig. 2B).

To determine if the CT production levels were strain- dependent, we also assessed the CT levels for *V. cholerae* CA401 and its Δ*nqr* deletion derivative, which, like O395N1, is another classical strain. Similar patterns were observed in the CA401 strain at both the early and late-log phases (Fig. 2C and 2D). Thus, in both strains the lack of NQR initially increased CT production but resulted in an overall decrease of CT production at the late-log growth phase despite the observation that an *nqr* deletion results in higher overall *toxT* production.

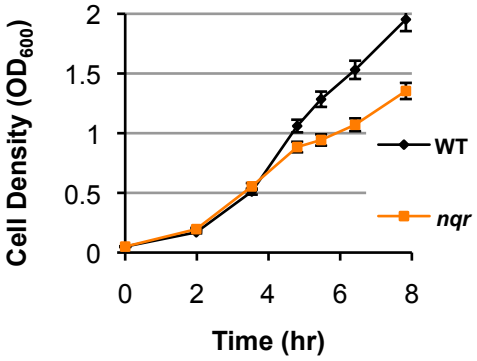
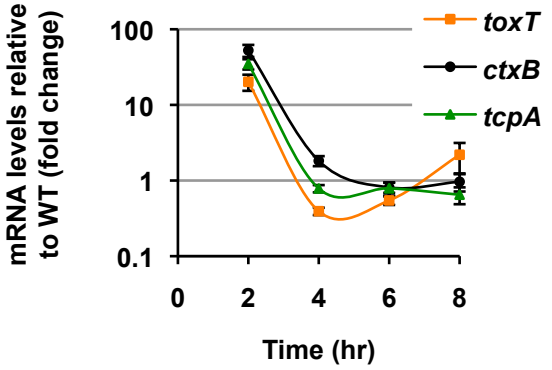


Figure 2A



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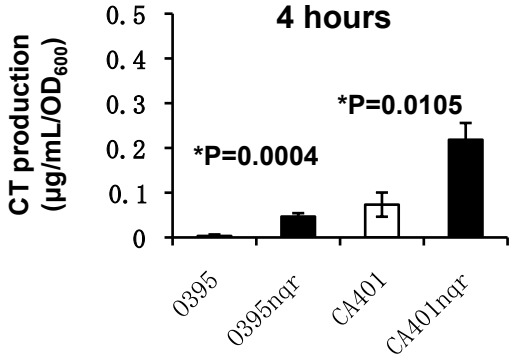

Figure 2C

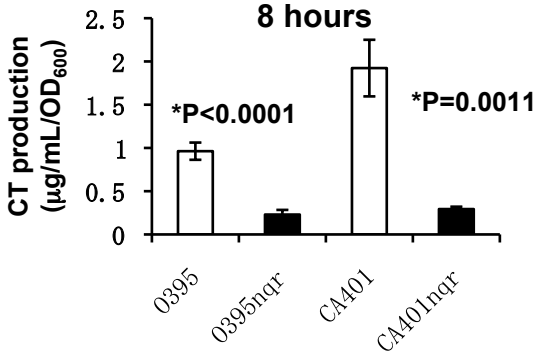

Figure 2D

Figure 2. Growth phase dependent virulence gene expression and CT production. Overnight growth cultures of the *V. cholerae* O395N1 and CA401 WT and the Δ*nqr* mutants were washed and diluted in LB (initial pH 6.5) to get OD_{600} = 0.05 and shaken in LB (initial pH 6.5) at 30°C in a roller drum. **(A)** Bacterial growth was measured by OD_{600}. The results shown are representative of at least three independent experiments. Error bars represent standard deviations. **(B)** Total RNA was extracted and analyzed by qRT-PCR. Gene expression levels were normalized using 16S ribosomal RNA. All experiments were repeated three times. The error bars indicate standard deviations. **(C, D)** The cell-free culture supernatants were prepared from 4 hour growth **(C)** and 8 hour growth **(D)** and assayed for CT production by CT-ELISA. Error bars represent standard deviations and P-values were obtained using Student's T-test.

These sets of experiments led to the observation that virulence gene expression and CT production are growth phase dependent. At the same time the Δ*nqr* mutant transitioned to slower growth at the mid-log growth phase (4 hours), virulence gene expression also decreased to WT levels (Fig. 2A, 2B). This suggests that virulence gene expression occurs earlier in the growth phase of the Δ*nqr* mutant than in the WT. Similarly, CT production was also elevated compared to the WT only at the early phases of growth (Fig. 2C, 2D). This observation coincides with the growth time-point data and the virulence gene mRNA levels.

NQR affects *toxT* expression via respiration activity

NQR has three main functions: NADH dehydrogenase, sodium pumping, and donating electrons to the quinone pool. We had previously proposed a connection between expression of the main *V. cholerae* virulence factors and sodium membrane bioenergetics on the basis of the finding that disruption of NQR activated *toxT* transcription (25). Further characterization of the link between the SMF and *toxT* expression, however, did not support this theory. First, *toxT* transcription was minimally affected by mutation of other SMF generating enzymes (e.g. the sodium/proton antiporters NhaA, and NhaD) (Fig. 3). Second, the NQR effect on *toxT* expression was found to be independent of the SMF-dependent *V. cholerae* flagella system (28). Thus, we concluded that SMF production *per se* is probably not directly linked to virulence gene expression.

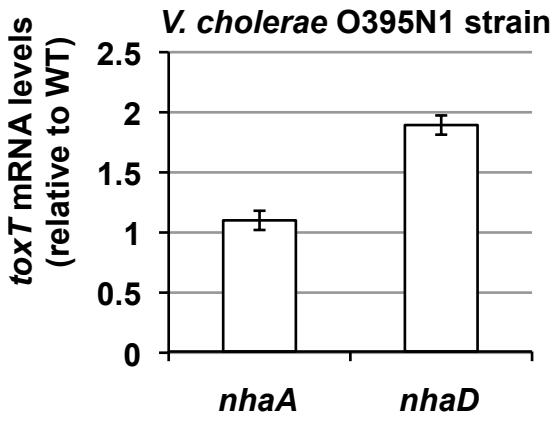

Figure 3. Effect of *nhaA* and *nhaD* mutations on *toxT* expression. *V. cholerae* O395N1 strain and the Δ*nhaA* and Δ*nhaD* mutants were grown in LB (initial pH 6.5) at 30°C. Total RNA was extracted at 6 hours and analyzed by qRT-PCR. Gene expression levels were normalized using 16S ribosomal RNA. All experiments were repeated twice. The error bars indicate standard deviations.

Because *V. cholerae* does not encode a Complex I ortholog, NQR is expected to also serve as the main respiratory enzyme. Loss of NQR should therefore decrease aerobic respiration activity. L-lactate is known to stimulate the ETC by inducing L-lactate-ubiquinone oxidoreductase activity and since *V. cholerae* is predicted to encode an inducible L-lactate-ubiquinone oxidoreductase enzyme (VCA0984), the addition of L-lactate to the growth medium should restore growth and virulence expression of the Δ*nqr* mutant. Incubation of the Δ*nqr* mutant with LB supplemented with 40mM L-lactate did restore growth closer to WT levels (Fig. 4). L-lactate also returned *toxT* transcription levels back to parental levels, as assessed by a β-galactosidase assay (Fig. 5). Based on our findings that SMF is probably not responsible for the changes in virulence gene expression, combined with the observation that L-lactate restores *toxT* transcription to

WT levels, we predict that respiration, rather than SMF, is responsible for the effects of the Δ*nqr* mutation on *toxT* transcription.

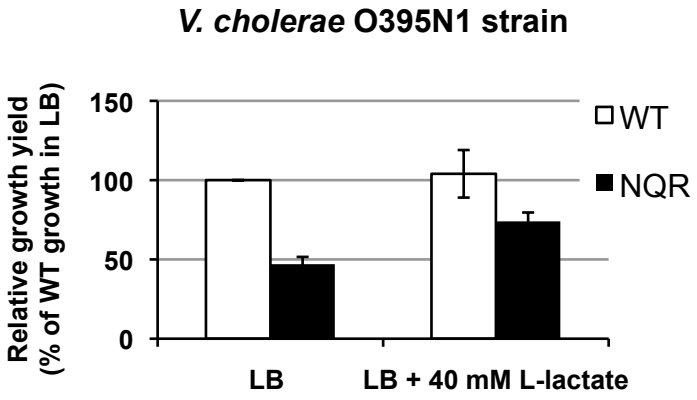

Figure 4. Effect of L-lactate on *V. cholerae* growth. Overnight growth cultures of *V. cholerae* O395N1 were washed and inoculated into LB (initial pH 6.5) with or without 40mM L-lactate and shaken at 30°C. L-lactate was also added to the pre-culture to induce L-lactate dehydrogenase. Growth yield was measured after 6 hours growth. All experiments were repeated more than three times. The error bars indicate standard deviations.

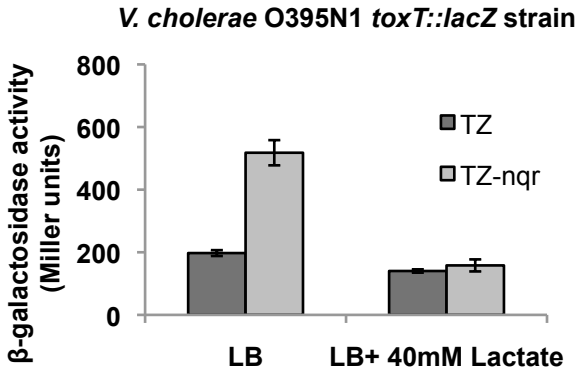

Figure 5. Effect of L-lactate on *toxT* expression. Overnight growth cultures of *V. cholerae toxT::lacZ* (TZ) and *toxT::lacZ* Δ*nqr* (TZ *nqr*) were washed and inoculated into LB (initial pH 6.5) with or without 40mM L-lactate and shaken at 30°C. The starting OD_{600} was adjusted to 0.05. The β-galactosidase activities were measured after 6 hours growth. L-lactate was also added to the pre-culture to induce L-lactate dehydrogenase. The error bars indicate standard deviations.

Additionally, we found that L-lactate restored growth of the Δ*nqr* mutant even in the presence of high NaCl (500mM) (Fig. 6). Growth of a Na^+/H^+ antiporter mutant, Δ*nhaA* was normal and not affected by the addition of L-lactate at either of the tested pH levels (6.5 and 8.5). However, growth of the combination mutant Δ*nqr-nhaA* was not fully restored by L-lactate, particularly at pH 8.5. These results indicate that the combination of the sodium pumps NQR and NhaA is essential for NaCl resistance.

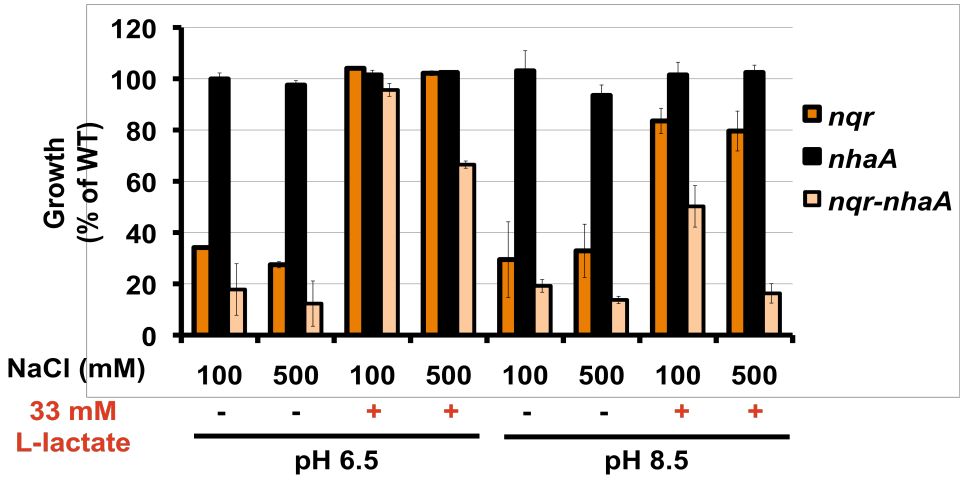

Figure 6. The effect of L-Lactate on *V. cholerae* growth in high NaCl media. *V. cholerae* O395N1 WT, Δ*nqr,* Δ*nhaA*, and Δ*nqr-nhaA* mutants were used. Overnight growth cultures of bacteria were washed and inoculated into LB (initial pH 6.5 or 8.5) with varying concentrations of NaCl (either 100mM or 500mM) and with or without 33 mM L-lactate and shaken at 37°C. The starting OD_{600} was adjusted to 0.05. L-lactate was also added to the pre-culture to induce L-lactate dehydrogenase. OD_{600} was measured after 16 hours and standardized to WT growth levels. The error bars indicate standard deviations.

In addition to restoring growth and *toxT* transcription, we found that the addition of 40 mM L-lactate into the LB also recovered CT production in the Δ*nqr* mutant to the parent strain level (Fig. 7). This suggests that reduced ETC activity is responsible for the

decreased CT production in the Δ*nqr* mutant. Furthermore, blocking ETC with either an NQR specific inhibitor, 2-n-Heptyl-4-hydroxyquinoline N-oxide (HQNO), or a succinate dehydrogenase inhibitor, thenoyltrifluoroacetone (TTFA), repressed extracellular CT production in both *V. cholerae* O395N1 and CA401 (Fig. 8). HQNO and TTFA also repressed CT production in an El Tor biotype strain, N16961, when tested in the El Tor CT stimulating growth conditions, known as the "AKI growth conditions" (31) (Fig. 7). These data further support the notion that inhibition of the ETC blocks virulence expression in *V. cholerae* and respiration, rather than SMF, is the probable mechanism through which the Δ*nqr* mutant modulates virulence gene expression.

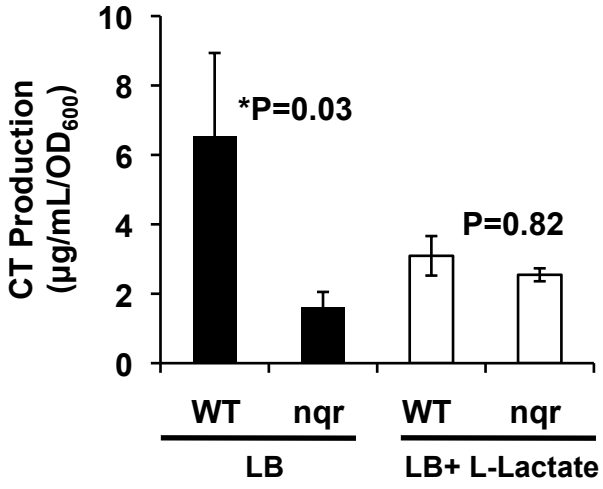

Figure 7. Effect of L-lactate on CT production. Overnight growth cultures of *V. cholerae* O395N1 WT and Δ*nqr* were washed and diluted in LB (initial pH 6.5) to starting OD_{600} = 0.05 and shaken at 30°C for 8 hours in a roller drum. The cell-free culture supernatants were prepared after growth and assayed for CT production by CT-ELISA. All experiments were repeated three times. The error bars indicate standard deviations and P-values were calculated using Student's T-test.

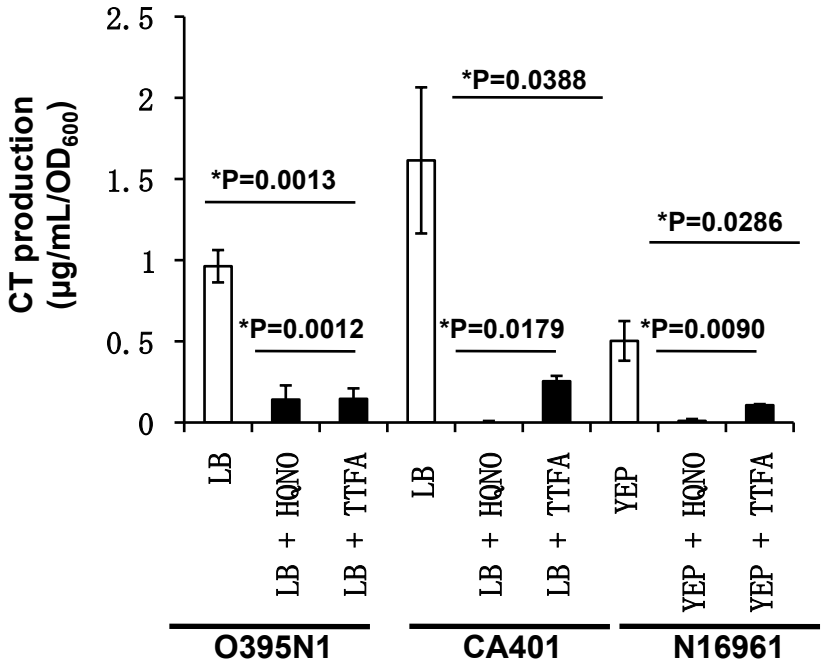

Figure 7. Effects of HQNO and TTFA on CT production. Overnight growth cultures of *V. cholerae* O395N1 and CA401 were washed and diluted in LB (initial pH 6.5) to starting OD_{600} = 0.05 and shaken at 30°C for 8 hours in a roller drum. Overnight growth cultures of *V. cholerae* N16961 were grown in YEP medium under AKI conditions. HQNO and TTFA were added at 2.5 μM. The cell-free culture supernatants were prepared after growth and assayed for CT production by CT-ELISA. All experiments were repeated three times. The error bars indicate standard deviations and P-values were calculated using Student's T-test.

Metabolism defects as detected by Phenotype Microarray™

Since NQR is expected to function as the main respiratory enzyme, we investigated how lack of NQR affects *V. cholerae* metabolism. To do this, we used Phenotype Microarray™ (Biolog) analysis to compare the metabolism of the Δ*nqr* mutant to that of its parent using PM1-PM10 microplates. The Phenotype Microarray™ is a high throughput screening system that allows for quantitative measurement of hundreds of carbon/nitrogen/sulfur/phosphorus sources at the same time. Cell suspension is inoculated into microwells, each containing a different sole carbon source. The phenotype is

measured using cell respiration as the universal reporter. If a phenotype is "strongly positive" the cells respire rapidly, reducing a tetrazolium dye and forming a strong color (35). The redox assay allows for both amplification and quantitative measurement of the phenotype.

Results from the PM plates showed that the Δ*nqr* mutant had defects in the utilization of 15 of the 190 carbon sources tested with the PM1 and PM2 microplates (Table 1). These 15 carbon sources included several Tricarboxylic acid (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), suggesting that TCA cycle activity is low in the Δ*nqr* mutant compared to the parent strain (36). Because succinic acid was used as the carbon source in the PM3-PM8 (nitrogen source, phosphorus source, sulfur sources and nutrient supplements plates), systematic metabolic defects were observed due to the inability of the Δ*nqr* mutant to utilize succinic acid. The use of succinic acid in the PM3-PM8 plates therefore made the effects of the Δ*nqr* mutation on metabolism uncertain so we tested those plates again using pyruvate as the sole carbon source. Pyruvate is one of the carbon sources that the Δ*nqr* mutant utilizes similarly to the parent strain. The results of the phenotype microarray indicated that the Δ*nqr* mutant strain still showed multiple defects in nitrogen, phosphate and sulfur utilization (Table 1). Using PM9 and PM10 plates, we also tested the effects of osmolytes and pH and found that the Δ*nqr* mutant is sensitive to sodium chloride, sodium sulfate, ethylene glycol, and urea (Table 1). These data further confirmed that lack of NQR broadly affects *V. cholerae* metabolism.

Table 1. Metabolism lost* in the Δ*nqr* mutant based on Biolog measurements.

Carbon Source	**Metabolite Name**
Amino Acid	L-Aspartic acid, L-Proline, L-Histidine, L-Glutamine, L-Glutamic acid, Gly-Glu
Carboxylic Acid	Acetic acid, D, L-Malic acid, Fumaric acid, Propionic acid, Succinic acid, L-Malic acid, Mono-Methylsuccinate, Succinamic acid
Ester	Methylpyruvate
Nitrogen Source	**Metabolite Name**
Amino Acid	L-Glutamine, L-Aspartic acid , Glycine, L-Glutamic acid, L-Citrulline, L-Asparagine, L-Tryptophan, L-Histidine, L-Arginine, L-Proline
Osmotic Sensitivity	**Metabolite Name**
Ethylene Glycol	20%, 15%, 10%, 5% Ethylene Glycol
Sodium Sulfate	5%, 3%, 2% Sodium sulfate
Sodium Chloride	3%, 4% Sodium chloride
Urea	2%, 3% Urea
Phosphorous Source	**Metabolite Name**
Inorganic/ Organic	Hypophosphite, Dithiophosphate, Phosphate, Trimetaphosphate D-3-Phospho-Glyceric acid, Phosphoenol Pyruvate, Phosphocreatine, 6-Phospho-Gluconic acid, Phosphono Acetic acid, Thymidine 3`-Monophosphate, Triethyl Phosphate, O-Phospho-L-Serine, O-Phospho-L-Threonine, 2-Deoxy-D-Glucose 6-Phosphate, O-Phospho-L-Tyrosine, Thymidine 3`, 5`-Cyclic Monophosphate, Phospho-Glycolic acid

Sulfur Source	Metabolite Name
Inorganic/ Organic	Tetrathionate, Glutathione, Cystathionine, 1-Thio-b-D-Glucose, L-Cysteine, Gly-Met, 2-Hydroxyethane Sulfonic acid, Butane Sulfonic acid, Methane Sulfonic acid, Tetramethylene Sulfone, Taurine, p-Aminobenzene Sulfonic acid, L-Cysteine Sulfinic acid, D-Cysteine, S-Methyl-L-Cysteine, Thiourea, Hypotaurine, Cys-Gly, D,L-Ethionine, L-Methionine Sulfoxide, Lanthionine, L-Methionine, D-Methionine, N-Acetyl-D,L-Methionine

*Based on assessment provided by Biolog

Transcriptome analyses

We performed microarray analyses to investigate how lack of NQR broadly affects gene expression in *V. cholerae.* Since we found that the Δ*nqr* mutant increases virulence gene expression, including *toxT*, *ctxB* and *tcpA*, only at the early phase of the logarithmic growth (Fig. 2B), this suggests that the lack of NQR affects *V. cholerae* gene expressions differently at the different growth phases. To understand the overall pattern of gene expression in the Δ*nqr* mutant, we performed microarray analyses using RNA prepared from bacterial cultures at the early and late phases of logarithmic growth. Microarray data from the early logarithmic growth phase showed that 612 genes were up regulated (around a 2-fold increase compared to WT) and 660 genes were down regulated (around a 2-fold decrease compared to WT). At the late logarithmic growth phase, 119 genes were up regulated and 264 genes were down regulated. Consistent with our previous data, the virulence genes, including *toxT*, *tcpA-F* and *ctxB,* were up regulated in the Δ*nqr* mutant at the early logarithmic growth phase but not at the late logarithmic

growth phase. We found 31 genes that were commonly up regulated and 55 genes that were commonly down regulated in both growth phases (Table 2).

The *cadBA* genes showed the highest increases in gene expression levels in the Δ*nqr* mutant in both growth phases (Table 2). The *cadA* gene encodes lysine decarboxylase and *cadB* encodes the lysine/cadaverine antiporter. The expression of *cadBA* is regulated by a ToxR type transcriptional regulator, CadC (37) and a LysR type transcriptional regulator, AphB (38). However, expression of the other AphB regulated genes, such as *tcpP* and *nhaB* (38), were not affected by the Δ*nqr* mutation.

Several transporter genes including tripartite ATP-independent periplasmic (TRAP) transporters, *siaPQM* and *dctMQP*, were down regulated in the Δ*nqr* mutant strain (Table 2). It was recently reported that SiaPQM is a sodium-dependent sialic acid-specific TRAP transporter and is required for utilizing sialic acid as a sole carbon source (39, 40). Consistent with this, multiple genes encoding enzymes in the sialic acid degradation pathway, which convert sialic acid to fructose 6-phosphate, were also down regulated in the Δ*nqr* mutant. *dctMQP* was recently shown to be a C4-dicarboxylate-specific TRAP transporter partly responsible for utilization of *V. cholerae* C4-dicarboxylates, succinate, malate, and fumarate (39). Thus, it might be possible that the decreased utilization of succinate, malate, and fumarate by the Δ*nqr* mutant was simply caused by the decreased uptakes of these C4-dicarboxylates.

The most broadly affected genes were flagella genes, which were down regulated in Δ*nqr*. These findings are consistent with our previous findings that disruption of the SMF through inhibition of NQR negatively affects SMF-dependent flagella rotation.

However, we also previously found that the effect of NQR on virulence expression is likely independent of the flagella system (28).

Table 2. Gene regulation in the *Δnqr* mutant based on microarray analysis.

VC Number	Functions	Fold change (4 hour)	Fold change (8 hour)
Up regulated in the Δ*nqr* mutant			
VC0828	toxin co-regulated pilin	1.056 up	1.009 down
VC1456	cholera enterotoxin, B subunit	45.008 up	13.236 down
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	urocanate 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 IIABC component	1.732 up	2.479 up
VCA1046	mannitol-1-phosphate 5-dehydrogenase	1.997 up	1.712 up
Down regulated in the Δ*nqr* mutant			
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	RstA1 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	1.663 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 FlaG	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
VCA0836	hexapeptide repeat-containing acetyltransferase	1.722 down	1.679 down
VCA0847	inner membrane protein YjeH	2.362 down	1.638 down
VCA0848	GGDEF family protein	2.354 down	1.502 down
VCA0862	long-chain fatty acid transport protein	4.995 down	1.830 down
VCA0864	methyl-accepting chemotaxis protein	1.582 down	1.694 down

Acetate Switch

When TCA cycle activity is low (e.g. in respiration mutants), bacteria utilize alternative pathways to generate ATP and to recycle CoASH from acetyl-CoA (41, 42). One such pathway is the PTA-ACK pathway, which consists of phosphotransacetylase (PTA) and acetate kinase (ACK) (41- 44). Metabolic activity through the PTA-ACK pathway results in the excretion of acetate into the external medium (41). Since the Δ*nqr* mutant is predicted to have reduced TCA cycle function, we monitored acetate excretion during growth in LB and found that the Δ*nqr* mutant accumulated larger concentrations of acetate in the external environment than did the WT strain (36).

When grown in tryptone-based rich media, *E. coli* first produces acetate by using the PTA-ACK pathway but at the certain growth phase point, 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 (41). To further examine acetate production in the Δ*nqr* mutant, we measured acetate excretion levels during a time course. We found that between 6 and 8 hours of growth in LB media

the WT strain started to reduce external acetate excretion, indicating that *V. cholerae* has an acetate switch similar to *E. coli* (Fig. 8). Interestingly, the Δ*nqr* mutant did not show this phenotype and continued to accumulate acetate until the end of its growth phase (Fig. 8). This pattern is consistent with the Phenotype Microarray™ data that showed the Δ*nqr* mutant had a defect in acetate utilization (Table1).

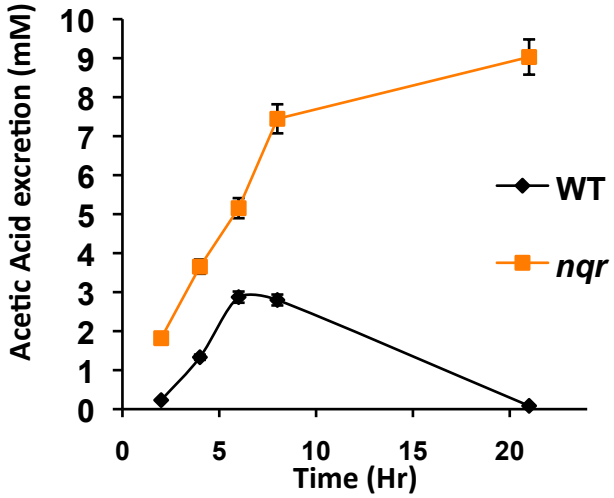

Figure 8. External acetate excretion. Overnight growth cultures of *V. cholerae* O395N1 WT and Δ*nqr* mutant were washed and inoculated into LB (initial pH 6.5) and shaken at 30°C in a roller drum. The starting OD_{600} was adjusted to 0.05. Acetate levels were measured in the cell-free supernatants at 2, 4, 6, 8, and 21 hours. All experiments were repeated 3 times. Error bars represent standard deviations.

Conclusions

Our goal was to characterize the role of NQR on *V. cholerae* virulence gene expression, metabolism, and physiology. Since NQR is expected to act as the main respiratory-linked sodium pump in *V. cholerae*, it should have an important role in the physiology and virulence gene expression of this organism. Indeed we did find that not only did the Δ*nqr* mutant have growth dependent defects compared to the WT but it also had broad defects in metabolism, as assessed by the phenotype microarray. Furthermore, we were able to conclude that the effects of NQR on *V. cholerae* virulence gene expression were linked to respiration rather than SMF. Microarray analysis further confirmed the growth phase dependent expression of virulence genes in the Δ*nqr* mutant along with TRAP genes, flagella genes, and several other physiologically important genes. Deregulation of the acetate switch in the Δ*nqr* mutant also suggests that the changes in metabolism caused by the inhibition of *nqr* are linked to increased virulence gene expression early in the growth phase but may correspond to the growth defects and lowered virulence gene expression later in the growth phase. With our findings, we conclude that NQR has broad, growth-dependent effects on *V. cholerae* metabolism and the observed changes in metabolic function are linked to the observed changes in virulence gene expression following loss of NQR.

While the majority of this work has been preliminary research to increase our understanding of NQR and the link between respiration, metabolism, and virulence gene expression in *V. cholerae*, much of this research has the potential to lead to exciting new avenues in our understanding of virulence in *V. cholerae*. In fact, our findings have recently led us to propose NQR as a novel drug target to treat cholera symptoms. NQR makes an intriguing drug target since it is only found in *V. cholerae* cells and human mitochondria do

not possess an NQR ortholog. In addition, both CT and TCP production are decreased in the Δ*nqr* mutant. The characterization of the role of NQR has been an important project to better understand how *V. cholerae* establishes infection and the role metabolism and respiration play in infection and will be an important foundation for future work.

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