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Inhibition of the sodium-translocating NADH-ubiquinone oxidoreductase [Na^+-NQR] decreases cholera toxin production in *Vibrio cholerae* O1 at the late exponential growth phase.

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Abstract

Two virulence factors produced by *Vibrio cholerae*, cholera toxin (CT) and toxin-corregulated pilus (TCP), are indispensable for cholera infection. ToxT is the central regulatory protein involved in activation of CT and TCP expression. We previously reported that lack of a respiration-linked sodium-translocating NADH–ubiquinone oxidoreductase (Na^+-NQR) significantly increases *toxT* transcription. In this study, we further characterized this link and found that Na^+-NQR affects *toxT* expression only at the early-log growth phase, whereas lack of Na^+-NQR decreases CT production after the mid-log growth phase. Such decreased CT production was independent of *toxT* and *ctxB* transcription. Supplementing a respiratory substrate, L-lactate, into the growth media restored CT production in the *nqrA-F* mutant, suggesting that decreased CT production in the Na^+-NQR mutant is dependent on electron transport chain (ETC) activity. This notion was supported by the observations that two chemical inhibitors, a Na^+-NQR specific inhibitor 2-n-Heptyl-4-hydroxyquinoline N-oxide (HQNO) and a succinate dehydrogenase (SDH) inhibitor, thenoyltrifluoroacetone (TTFA), strongly inhibited CT production in both classical and El Tor biotype strains of *V. cholerae*. Accordingly, we propose the main respiratory enzyme of *V. cholerae*, as a potential drug target to treat cholera because human mitochondria do not contain Na^+-NQR orthologs.

1. Introduction

Vibrio cholerae is the etiological agent of cholera, a life-threatening diarrheal disease. Toxin-coregulated pilus (TCP) and cholera toxin (CT) are critical determinants of the pathogenicity of *V. cholerae*. TCP is a Type IV pilus that is required for colonization in the small intestine [1], whereas CT is a secreted enterotoxin responsible for inducing severe watery diarrhea, a hallmark feature of cholera [2]. The expression levels of TCP and CT are positively regulated by an AraC-type transcriptional regulator, ToxT [3].

The sodium-translocating NADH–ubiquinone oxidoreductase (Na^+-NQR) is a unique redox-driven sodium pump and is found in the electron transport chain (ETC) of a number of pathogenic and marine bacteria [4]. Na^+-NQR is predicted to play a vital role in the ETC of these organisms, including *V. cholerae*, that do not possess an ortholog of the mitochondrial Complex I, which is typically the main ETC-linked NADH-dehydrogenase (http://gsc.jcvi.org/projects/msc/vibrio/). It is well known that the genes encoding NQR are strongly repressed at anaerobic conditions [5,6]. Since some parts of the small intestine are anaerobic, one might speculate that lack of Na^+-NQR does not affect *V. cholerae* O1 virulence. However, a previous study revealed that Na^+-NQR is essential for *V. cholerae* O1 colonization in the small intestine of mice and in acid tolerance response (ATR) [7]. This suggested that Na^+-NQR is essential for *V. cholerae* O1 virulence and could be used as a molecular target to develop new therapeutic treatments for cholera. In this study, we further aimed to examine the link between Na^+-NQR and virulence factor production as a first step to evaluate Na^+-NQR as a molecular target for anti-cholera drug development.

2. Materials and Methods

2.1. Bacterial strains, plasmids and media. *V. cholerae* O1 classical biotype strains, O395N1 and CA401, their Δ*nqrA-F* mutant strains, and El Tor biotype strain, N16961, were used in this study. The Δ*nhaA* mutant, the Δ*nhaB* mutant and the Δ*mrpA-F* mutant strains of *V. cholerae* O395N1 (Quinn et. al. unpublished) were also used in this study. All bacterial strains were kept at -80°C in 20% glycerol stocks. The classical biotype strains were grown overnight in Luria-Bertani (LB) medium (Difco) at 37°C, washed, diluted to OD_{600} = 0.05 in LB (initial pH 6.5), and grown at 30°C. The pH of the LB medium was adjusted to pH 6.5 with HCl. The El Tor biotype strain, N16961, was grown overnight in LB medium at 37°C and then grown in Yeast Extract Peptone water (YEP) as described previously (i.e., AKI growth conditions) [8]. HQNO and TTFA were added at 2.5 μM. L-lactate was added at 40 mM. L-lactate was also added to the pre-cultures to induce L-lactate dehydrogenase activity. Streptomycin was supplemented at 100 μg/ml.

2.2 Quantitative reverse transcription-polymerase chain reaction (qRT-PCR) analysis. Cells of *V. cholerae* O1 grown in LB (initial pH 6.5) at 30°C for 2, 4, 6, and 8 hours were treated with RNA Protect Bacteria Reagent (Qiagen). RNA was extracted using the QIAGEN RNeasy Mini Kit (Qiagen) and treated with TURBO DNA-free™ Kit (Invitrogen). Primers used for qRT-PCR are 5Vc16SrRNAqRT: GATCATGGCTCAGATTGAACG, 3Vc16SrRNAqRT: TCGCCACCCAAGGAACA, 5VcToxTqRT: GCTGTCCTTTCTGAAGTGGTAAATG, 3VCToxTqRT: TTCTACTTTCGAGAAGAACCCTGAA, 5VcCtxBqRT: AGCGATTGAAAGGATGAAGGA, 3VcCtxBqRT: CGCATGAGGCGTTTTATTATTC, 5VcTcpAqRT:

CGTAATGCAGCAGCTAATAAAGCA, 3VcTcpAqRT: GGAACATATCACCGACACTGGTAA. Real-time qRT-PCR reactions were performed using the SuperScript® III Platinum® SYBR® Green One-Step qRT-PCR Kit (Invitrogen) and an ABI PRISM 7500 FAST Sequence Detection System (Applied Biosystems) at the Center for Genome Research and Biocomputing Core Laboratory at Oregon State University.

2.3 Measurements of CT production. CT production was determined by GM_1-based enzyme linked immunosorbent assays (CT-ELISA) essentially as described [9]. In brief, CT-ELISA was performed using a cholera toxin-specific monoclonal antibody (Abcam) and Goat-Anti-Mouse (GAM)-HRP Conjugated antibodies (Bio-Rad). An HRP Substrate kit (Bio-Rad) was used to detect the HRP activity and the plates were read at 415 nm on an iMark microplate reader (Bio-Rad). The amount of CT was quantified using known amounts of purified cholera toxin B subunit (Sigma) as the standard.

3. Results

3.1 Growth phase dependent effects of Na^+-NQR on toxT *expression and cholera toxin production.* Because we previously reported that Na^+-NQR affects *toxT* transcription [10], we monitored the growth and virulence gene expressions using *V. cholerae* parent and isogenic O395N1Δ*nqrA-F* mutant strains cultured under conditions typically used for *in vitro* induction of virulence gene expression [LB (initial pH 6.5) at 30°C] [9]. Initially, both strains displayed very similar growth rates, although the O395N1Δ*nqrA-F* mutant transitioned to a slower growth rate starting approximately from the mid- to late-exponential growth phase (Fig. 1A). Measurements of *toxT*, *ctxB* and *tcpA* expression levels in the O395N1Δ*nqrA-F* mutant were compared with the parent

strain by qRT-PCR. Consistent with our previous findings [10], the O395N1Δ*nqrA-F* mutant showed higher *toxT*, *ctxB* and *tcpA* expression levels than the isogenic parent strain, however, this effect was only observed at the very early exponential growth phase (2 hr growth) (Fig. 1B). In agreement with the gene expression data, extracellular CT levels in the O395N1Δ*nqrA-F* mutant were higher than in the parent strain at the early exponential growth phase (Fig. 1C). In contrast, the O395N1Δ*nqrA-F* mutant showed significantly lower extracellular CT levels compared to the parent strain at the late exponential growth phase (Fig. 1D), even though transcriptional levels of the *toxT* and *ctxB* genes were similar in both strains during late exponential growth phase (6 hr and 8 hr growth) (Fig. 1B). Similar extracellular CT level patterns were also observed in another *V. cholerae* O1 classical strain, CA401 and its Δ*nqrA-F* deletion derivative (Fig. 1C and 1D).

The autoagglutination phenotype is correlated with TCP production [11]. Here, we observed that both the O395N1Δ*nqrA-F* and the CA401Δ*nqrA-F* strains did not show a full autoagglutination phenotype after overnight growth in LB (initial pH 6.5) at 30°C, whereas both of the parent strains did (data not shown). This suggested that the lack of functional Na^+-NQR also negatively affects overall TCP production, although *tcpA* transcription levels were comparable between the O395N1 parent and Δ*nqrA-F* mutant strains (Fig. 1B).

3.2 Other sodium-translocating enzymes do not affect CT production. We next aimed to gain a better understanding of the effects of loss of Na^+-NQR on CT production in *V. cholerae* O1. The Na^+-NQR has two major functions: it is a primary sodium pump and a major component in the *V. cholerae* ETC [4]. To examine the role

of sodium pumping, we investigated the effects of loss of other sodium-translocating enzymes on CT production. Genetic inactivation of various sodium/proton antiporters, including NhaA, NhaD, and Mrp, did not have any effects on extracellular CT levels (data not shown), suggesting that loss of sodium pumping *per se* does not affect extracellular CT levels. Furthermore, we noted that the addition of L-lactate into the growth media "rescued" extracellular CT levels in the O395N1Δ*nqrA-F* mutant to the levels observed in the parent strain (Fig. 2). Since L-lactate is a respiration substrate for the L-lactate-ubiquinone oxidoreductase activity, these data suggested that reduced ETC activity might be responsible for the decreased extracellular CT levels in the *V. cholerae* O1 Na^+-NQR mutants.

3.3 ETC inhibitors inhibit CT production. To further investigate the role of ETC on CT production, we investigated the effects of the Na^+-NQR-specific inhibitor, 2-n-Heptyl-4-hydroxyquinoline N-oxide (HQNO), and a succinate dehydrogenase (SDH) inhibitor, thenoyltrifluoroacetone (TTFA), on CT production. In agreement with the data from the O395N1Δ*nqrA-F* and the CA401Δ*nqrA-F* strains, HQNO inhibited CT production in two classical biotype wild-type strains of *V. cholerae* O1, O395N1 and CA401 (Fig. 3). Similarly, TTFA also inhibited CT production in these two strains.

To investigate whether ETC inhibitors also inhibited CT production in an El Tor biotype strain, we tested the effect of these chemicals on CT production of *V. cholerae* N16961. When grown under AKI conditions, a specific growth condition for the El Tor biotype strain to produce measurable CT *in vitro* [8], the *V. cholerae* N16961 strain produced detectable amounts of CT as expected. However, addition of HQNO or TTFA to the growth media strongly inhibited CT production of *V. cholerae* N16961 (Fig. 3).

These data suggested that the ETC activities are essential for CT production in both classical and El Tor biotype strains.

4. Discussion

In this study, we found that inhibition of *V. cholerae* O1 Na^+-NQR inhibited CT production. Further studies revealed that such decreased CT production is not a Na^+-NQR specific effect and inhibition of other ETC components, such as SDH, also inhibited CT production. In general, many of the ETC inhibitors are highly toxic to human cells due to their inhibitory effect on the mitochondrial ETC [12]. Thus, for future antimicrobial drug development, it is important to target bacterial ETC components that do not exist in the human mitochondrial ETC. Comparison of the *V. cholerae* and the human mitochondrial ETCs revealed that Na^+-NQR, two *bd*-type oxidases and three ETC-linked dehydrogenases (DHs) are present in *V. cholerae* that are not in found the human mitochondrial ETC (Fig. 4). Thus, apart from Na^+-NQR, such *bd*-type oxidases and ET- linked DHs might also be potential drug targets to inhibit CT and TCP production in *V. cholerae* O1. We are currently investigating this possibility.

We found that the *V. cholerae* O1 Δ*nqrA-F* mutant showed growth defects when grown in LB media. Similar growth kinetics have previously been observed for an *Escherichia coli* Complex I (NADH:ubiquinone oxidoreductase, *nuoB*) mutant in tryptone medium [13]. Furthermore, like the *E. coli nuoB* mutant [13], the O395N1Δ*nqrA-F* mutant showed decreased acetate utilization [14], suggesting a depletion of available NAD^+. Taken together with the fact that the *V. cholerae* genome does not encode *nuo* genes, these observations suggested that Na^+-NQR is the main ETC-linked NADH dehydrogenase in *V. cholerae*. Similar to *V. cholerae*, some other important pathogenic

bacteria, such as *Vibrio parahaemolyticus*, *Vibrio vulnificus*, and *Haemophilus influenza* do not have Nuo but instead have Na^+-NQR, suggesting that Na^+-NQR plays major roles in the ETC in these pathogenic bacteria. On the other hand, some pathogenic bacteria, such as *Yersinia pestis* and *Pseudomonas aeruginosa*, have both Na^+-NQR and Nuo. In contrast to *V. cholerae*, the *Y. pestis* and *P. aeruginosa* strains that lack functional Na^+-NQR did not show growth defects when grown in LB media (Minato et.al. unpublished data), suggesting that lack of Na^+-NQR significantly affects bacterial ETC activity only when Nuo is absent. It is also important to note that lack of Na^+-NQR does not affect *Y. pestis* virulence [15].

The temporal increase in *toxT* expression in the *V. cholerae* O1 Δ*nqrA-F* mutant might be caused by a combination of multiple factors. Our recent study suggested that lack of Na^+-NQR increases *toxT* expression via affecting acetyl-CoA [14]. Interestingly, it was known that intracellular acetyl-CoA levels are higher only at the early-log growth in *E. coli* [16]. Thus, it is tempting to speculate that lack of Na^+-NQR increases *toxT* expression only at the early-log growth phase because intracellular acetyl-CoA levels are high only at the early-log growth phase.

The overall CT production of the *V. cholerae* O1 Δ*nqrA-F* mutant was much less than the parent strain. Since *ctxB* gene expressions in the Δ*nqrA-F* mutant were similar to the parent strain after the mid-log growth phase, these data indicated that lack of Na^+-NQR has a negative impact on CT levels via affecting either translation or secretion. Future studies will be necessary for understanding the molecular mechanism of Na^+-NQR mediated CT production.

In summary, our data raised the possibility of using Na^+-NQR as a potential novel molecular target for the development of new therapeutic interventions against cholera for several reasons: 1) Na^+-NQR appears to be the major ETC-linked enzyme in *V. cholerae*; 2) genetic or chemical inactivation of Na^+-NQR significantly diminishes the overall levels of CT; 3) the *V. cholerae* Δ*nqrA-F* mutant has a severe to moderate growth defect; 4) the *V. cholerae* Δ*nqrA-F* mutant was previously found to be attenuated in a mouse model [7]; and 5) Na^+-NQR has no orthologs in the human cells. Because inhibition of Na^+-NQR does not kill bacteria, targeting Na^+-NQR would be expected to produce less pressure to evolve bacterial resistance compared to the traditional antimicrobial agents, similar to the other "anti-virulence" drug strategies [17].

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Figure legends

Figure 1. Growth-phase dependent virulence gene expression and CT production. Overnight cultures of *V. cholerae* O395N1, CA401, and their respective isogenic Δ*nqrA-F* mutants were washed and diluted in LB (initial pH 6.5) to OD_{600} = 0.05 and shaken in LB (initial pH 6.5) at 30°C. All experiments were repeated three times. The error bars indicate standard deviations. (A) Bacterial growth was measured by OD_{600}. (B). Total RNA was extracted and analyzed by qRT-PCR. Gene expression levels were normalized between the samples by using 16S ribosomal RNA. (C, D) The cell-free culture supernatants were prepared from 4hr growth (C) and 8hr growth (D) and assayed for CT production by CT-ELISA. P values were calculated by Student's t test and * indicates $P< 0.05$.

Figure 2. The effects of L-lactate on CT production. Overnight cultures of *V. cholerae* O395N1 and its respective isogenic Δ*nqrA-F* mutant were washed and diluted in LB (initial pH 6.5) to OD 600 = 0.05 and shaken in LB (initial pH 6.5) at 30°C for overnight. The cell-free culture supernatants were prepared after growth and assayed for CT production by CT-ELISA. All experiments were repeated at least three times. The error bars indicate standard errors. P values were calculated by one-way ANOVA followed by post hoc comparisons using the Bonferroni test and * indicates statistical significance ($P< 0.05$).

Figure 3. The effects of HQNO and TTFA on CT production. Overnight cultures of *V. cholerae* O395N1 and CA401 were washed and diluted in LB (initial pH 6.5) to the initial

294 OD_{600} = 0.05 and cultured in LB (initial pH 6.5) at 30°C for 8hr. Cultures of *V. cholerae*
295 N16961 were grown in YEP medium under AKI conditions. HQNO and TTFA were
296 added at 2.5 μM. The cell-free culture supernatants were prepared after growth and
297 assayed for CT production by CT-ELISA. All experiments were repeated three times.
298 The error bars indicate standard errors. P values were calculated by one-way ANOVA
299 followed by post hoc Bonferroni test and * indicates $P< 0.05$.
300
301 Figure. 4. Putative reductases and oxidases of ubiquinone (UQ) predicted from the *V.*
302 *cholerae* O395N1 and N16961 genomes are shown. Green represents the components
303 present both in *V. cholerae* and human ETC. Orange represents the components that
304 are not present in human ETC. The UQ reductase activity of NQR (VC2290-95) is
305 coupled by the NADH oxidation and sodium extrusion activity. The UQ reductase
306 activities of succinate dehydrogenase (SDH) (VC2088-91) and glycerol-3-phosphate
307 dehydrogenase (GlpD) (VCA0657) are coupled to the oxidation of succinate and
308 glycerol-3-P to fumarate and dehyroxyacetone-P, respectively. Other bacteria-specific
309 respiration-linked dehydrogenases [such as Ndh2 (VC1890), L-lactate dehydrogenase
310 (VCA0984), and D-amino acid dehydrogenase (VC0786)] are shown collectively (DHs).
311 The *bd*-type cytochrome (bd cyt) oxidase-1 (VC1843-44) and oxidase-2 (VCA0872-73)
312 oxidize the reduced form of UQ coupled to oxygen reduction and proton efflux activities.
313 The ubiquinol-cytochrome C reductase (Fe/S bc1) (VC0573-75) also oxidizes the
314 reduced form of UQ and the reaction is coupled with the cytochrome c4 (C) reduction
315 and proton efflux activities. The reduced form of C is oxidized by cytochrome C (cytC)
316 oxidase and the reaction is coupled to oxygen reduction and proton efflux activities.