The Na⁺-Translocating NADH:Quinone Oxidoreductase Enhances Oxidative Stress in the Cytoplasm of *Vibrio cholerae*

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ABSTRACT

We searched for a source of reactive oxygen species (ROS) in the cytoplasm of the human pathogen *Vibrio cholerae* and addressed the mechanism of ROS formation using the dye 2',7'-dichlorofluorescein diacetate (DCFH-DA) in respiring cells. By comparing *V. cholerae* strains with or without active Na⁺-translocating NADH:quinone oxidoreductase (Na⁺-NQR), this respiratory sodium ion redox pump was identified as a producer of ROS *in vivo*. The amount of cytoplasmic ROS detected in *V. cholerae* cells producing variants of Na⁺-NQR correlated well with rates of superoxide formation by the corresponding membrane fractions. Membranes from wild-type *V. cholerae* showed increased superoxide production activity (9.8 ± 0.6 μmol superoxide min⁻¹ mg⁻¹ membrane protein) compared to membranes from the mutant lacking Na⁺-NQR (0.18 ± 0.01 μmol min⁻¹ mg⁻¹). Overexpression of plasmid-encoded Na⁺-NQR in the *nqr* deletion strain resulted in a drastic increase in the formation of superoxide (42.6 ± 2.8 μmol min⁻¹ mg⁻¹). By analyzing a variant of Na⁺-NQR devoid of quinone reduction activity, we identified the reduced flavin adenine dinucleotide (FAD) cofactor of cytoplasmic NqrF subunit as the site for intracellular superoxide formation in *V. cholerae*. The impact of superoxide formation by the Na⁺-NQR on the virulence of *V. cholerae* is discussed.

IMPORTANCE

In several studies, it was demonstrated that the Na⁺-NQR in *V. cholerae* affects virulence in a yet unknown manner. We identified the reduced FAD cofactor in the NADH-oxidizing NqrF subunit of the Na⁺-NQR as the site of superoxide formation in the cytoplasm of *V. cholerae*. Our study provides the framework to understand how reactive oxygen species formed during respiration could participate in the regulated expression of virulence factors during the transition from aerobic to microaerophilic (intestinal) habitats. This hypothesis may turn out to be right for many other pathogens which, like *V. cholerae*, depend on the Na⁺-NQR as the sole electrogenic NADH dehydrogenase.

Vibrio cholerae is a Gram-negative bacterium and the causative agent of cholera, a devastating diarrheal disease. It is found in estuaries, inhabiting seawater or brackish water where it is commonly associated with biofilms on phyto- or zooplankton (1). The high salinity in these habitats is counteracted by an electrochemical sodium motive force (SMF) generated by the respiratory chain of *V. cholerae* (2, 3). This SMF drives cellular processes like substrate transport (4) and motility (5). It is generated by the Na⁺-translocating NADH:quinone oxidoreductase (Na⁺-NQR), which couples the energy that is released by oxidation of NADH with ubiquinone to the transport of sodium ions out of the cytoplasm with a 1e⁻/1Na⁺ stoichiometry (6). The Na⁺-NQR is a membrane-bound protein complex consisting of six subunits, NqrABCDEF, and contains a variety of different cofactors (3). Electron transfer from NADH to ubiquinone is mediated by a noncovalently bound flavin adenine dinucleotide (FAD) and a [2Fe-2S] cluster, both located in NqrF (7, 8), an iron center deeply embedded in the membrane-bound NqrD and NqrE subunits (3), a flavin mononucleotide (FMN) in NqrC (FMN_C) (9), an FMN and a riboflavin in NqrB (9–11), and finally a tightly bound ubiquinone-8 (Q8) acting as a reductant for the ubiquinone substrate. The localization of the ubiquinone binding site in Na⁺-NQR is controversial. Strickland and colleagues described a high-affinity quinone (Q) binding site on the NqrB subunit (12), whereas results from our group indicated binding of Q to the NqrA subunit (13). The recent three-dimensional (3D) structure of Na⁺-NQR revealed that FMN_C is the only cofactor in Na⁺-

NQR that is located in the periplasm and that all other cofactors are located in the cytoplasm or the transmembrane regions of the protein (3). It was observed that during substrate turnover, Na⁺-NQR produces superoxide *in vitro* (8), but the site of production within Na⁺-NQR has not been investigated. Superoxide formation has been extensively studied in the respiratory electrogenic NADH:quinone oxidoreductase found in mitochondria (complex I), which is not related to Na⁺-NQR (14, 15). The NADH binding pocket in complex I houses one FMN and a [2Fe-2S] cluster, which is reminiscent of the Na⁺-NQR nucleotide binding pocket on NqrF harboring FAD and a [2Fe-2S] cluster. To date, it is well accepted that the major fraction of superoxide produced by complex I is formed during electron transfer from the NADH to the [2Fe-2S] cluster via the FMN. The reduced FMN (FMN reduced by one electron [16] or two electrons [15]) reacts with dioxygen in

Received 22 April 2016 Accepted 5 June 2016

Accepted manuscript posted online 20 June 2016

Citation Muras V, Dogaru-Kinn P, Minato Y, Häse CC, Steuber J. 2016. The Na⁺-translocating NADH:quinone oxidoreductase enhances oxidative stress in the cytoplasm of *Vibrio cholerae*. J Bacteriol 198:2307–2317. doi:10.1128/JB.00342-16.

Editor: V. J. DiRita, Michigan State University

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Supplemental material for this article may be found at <http://dx.doi.org/10.1128/JB.00342-16>.

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the formation of superoxide. In a similar mechanism, superoxide could emerge in Na^+ -NQR, where FAD constitutes the primary electron acceptor and facilitates the one-electron transfer to the [2Fe-2S] cluster. However, in contrast to complex I, Na^+ -NQR contains three additional flavins which also could account for the generation of superoxide. To identify the site of superoxide production in *V. cholerae* (and in the Na^+ -NQR), we developed a protocol to detect cytoplasmic superoxide formation *in vivo* and studied superoxide production by wild-type Na^+ -NQR and its variant lacking one of the two flavin cofactors oriented to the cytoplasmic aspect of the Na^+ -NQR. This Na^+ -NQR variant retains NADH oxidation activity due to the remaining FAD in NqrF which is exposed to the cytoplasm *in vivo*. We show that Na^+ -NQR predominantly produces superoxide, and not hydrogen peroxide, and that the enzyme contributes to oxidative stress in the cytoplasm of *V. cholerae*. We propose that the FAD on NqrF represents the site of superoxide formation *in vivo*. The impact of Na^+ -NQR-dependent superoxide formation on signaling and virulence factor production in *V. cholerae* is discussed.

MATERIALS AND METHODS

Strains and plasmids. *Vibrio cholerae* O395N1 (17) and its variants devoid of Na^+ -NQR (*V. cholerae* Δnqr) (18) or regulator OxyR (*V. cholerae* ΔoxyR) were used. Strains were transformed with plasmid pNqr1 containing the six *nqrABCDEF* genes (19), or with plasmid pNmB coding for a variant of the Na^+ -NQR devoid of the NqrB subunit (11). Expression from both plasmids was induced by L-(+)-arabinose at the indicated concentrations.

V. cholerae ΔoxyR was constructed as described previously (20, 21). The antibiotic cassette (Cm) together with the flanking FLP recombination target (FRT) sites were amplified from plasmid pKD3, using the primers F-FRT (F stands for forward) (TGTTAGGCTGGAGCTGCTT) and R-FRT (R stands for reverse) (CATATGAATATCCTCCTTAG). Amplification of homologous regions flanking the target gene was performed with the following primer pairs: primers Fup-oxyR (TTTCATTATGGATCCTCAATCGG) and Rup-oxyR::FRT (AAGCAGCTCCAGCCTACACA CCAAGTATTCAAAATCACGAATGTTT) and primers Fdown-FRT::oxyR (CTAAGGAGGATATTCATATGGTAAGACGGCGAATCGAATACAAA) and Rdown-oxyR (ACTGAGAATTGGTTTAGCCCAATC), respectively. The amplification products were combined in a second PCR (primers Fup-oxyR and Rdown-oxyR), and the construct was used for transformation in electrocompetent *V. cholerae* O395N1. After selection, the antibiotic resistance cassette was removed with FLP recombinase, encoded on the temperature-sensitive plasmid pCP20. The helper plasmid was removed from the cells by growth at 42°C overnight. Antibiotic-sensitive colonies were identified by selection on agar plates, and gene replacement was confirmed by PCR analysis.

Growth of *V. cholerae* cells for analysis of superoxide formation. *V. cholerae* strains from cryocultures were spread on agar plates (1.5% [wt/vol] Bacto agar) prepared with LB_{glc} (1% [wt/vol] tryptone, 0.5% [wt/vol] yeast extract, 1% [wt/vol] sodium chloride, 10 mM glucose, and 50 mM potassium phosphate [pH 8.5]) and cultivated aerobically at 37°C overnight. Media contained 50 $\mu\text{g ml}^{-1}$ streptomycin and also 200 $\mu\text{g ml}^{-1}$ ampicillin for strains transformed with pNqr1 or pNmB. In order to monitor formation of superoxide by respiring *V. cholerae*, one colony was picked and used to inoculate 5 ml of LB_{glc} . The cells were cultivated aerobically overnight (37°C, 180 rpm). On the next day, 500 μl was used to inoculate 50 ml LB_{glc} , and growth was continued at 37°C for 3 h. With strains transformed with plasmids, 6.5 mM L-(+)-arabinose was added to induce the expression of Na^+ -NQR variants after an optical density at 600 nm (OD_{600}) of 1.0 was reached (after approximately 3 h). Growth was continued for 1 h. Cells from aliquots of 10 ml were harvested by centrifugation (8,500 rpm on a GSA rotor; Sorvall) at 4°C. The cell pellets were suspended in 40 mM Na_2SO_4 , 10 mM glucose, 20 mM $(\text{NH}_4)_2\text{SO}_4$, and 40

mM Tris- H_2SO_4 (pH 7.1) to obtain an OD_{600} of 10. The cell suspensions were used immediately for analysis of superoxide formation.

Fluorescence-based detection of reactive oxygen species (ROS) using DCFH-DA. To monitor superoxide-induced formation of dichlorofluorescein (DCF) by respiring *V. cholerae* cells, 96-well plates (96-well Nunc MicroWell plates with Nunclon delta surface; Thermo Fisher Scientific Inc.) with reservoirs filled with 1.7 ml double-distilled water (ddH_2O) were used to minimize evaporation of H_2O from the cell suspensions. To each well, 70 μl of ddH_2O , 10 μl of 10 $\times$ buffer (180 mM Tris- H_2SO_4 [pH 7.1], 450 mM Na_2SO_4 , 0.45 mg ml^{-1} bovine serum albumin [BSA], and 10 μl of 150 $\mu\text{g ml}^{-1}$ dichlorodihydrofluorescein diacetate [DCFH-DA] [final concentration, 15 $\mu\text{g ml}^{-1}$]) were added. Then, 10 μl of the *V. cholerae* cell suspension was added to each well to start the reaction. Inhibition studies were carried out with 2-*n*-heptyl-4-hydroxyquinoline (HQNO), which is a specific inhibitor of the Na^+ -NQR (22). HQNO was added from a 50 μM aqueous stock solution to a final concentration of 5 μM . In this case, only 60 μl ddH_2O was added to each well. The plates were incubated in the dark at 37°C at 60 rpm in an orbital incubator (Sartorius Stedim Biotech). After 16 h, DCF formation was determined from the fluorescein emission at 535 nm (bandwidth, 20 nm) using a Tecan Infinite F200 Pro plate reader (25 reads per well; integration time, 20 μs). Time-dependent analyses in the Tecan Infinite F200 Pro plate reader were performed using either i-control software or Magellan software. For measurements performed with i-control software, plates were incubated in an orbital shaker (37°C, 60 rpm) and placed in the plate reader prior to measurements. Measurements performed with Magellan software allowed shaking of plates in the plate reader (141 rpm) during a total incubation time of 24 h at 37°C.

To monitor nonspecific transformation of DCFH-DA to a fluorophore in the absence of cells, 10 μl DCFH-DA (150 $\mu\text{g ml}^{-1}$) was added to 90 μl of a glucose-containing, salt-based minimal medium (47.7 mM $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$, 22 mM KH_2PO_4 , 8.5 mM NaCl, 18.7 mM NH_4Cl , 2 mM MgSO_4 , 0.1 mM CaCl_2 with 22 mM glucose), LB adjusted to pH 7.1 with NaOH, or a buffer devoid of a carbon source (45 mM Na_2SO_4 , and 0.045 mg ml^{-1} BSA, 18 mM Tris- H_2SO_4 [pH 7.1]).

To investigate the effects of various oxygen concentrations on DCFH-DA fluorescence, the fluorescence emission spectra of 15 $\mu\text{g ml}^{-1}$ DCFH-DA in 18 mM Tris- H_2SO_4 (pH 7.1), 45 mM Na_2SO_4 , 0.045 mg ml^{-1} BSA (excitation wavelength, 485 nm; bandwidth, 10 nm) were recorded on a Horiba fluorospectrophotometer (Fluorolog) before and after removal of O_2 . The cuvette was sealed with a rubber septum, and the cuvette was repeatedly evacuated and flushed with N_2 by stirring with a magnetic bar to reduce partial oxygen pressure (<5 ppm of O_2 by volume).

Viability and survival of *V. cholerae* in the presence of DCFH-DA. *V. cholerae* cells were mixed with assay buffer and 15 $\mu\text{g ml}^{-1}$ DCFH-DA in 96-well plates for analysis of superoxide formation as described above. Immediately after adding DCFH-DA and after 16 h of incubation, aliquots of 20 μl were mixed with phosphate-buffered saline (PBS) (136 mM NaCl, 2.7 mM KCl, 12.5 mM Na_2HPO_4 , 2 mM KH_2PO_4 [pH 7.1]) to obtain dilutions of the cell suspension from 10^{-1} to 10^{-10} . From each dilution, drops of 20 μl were applied to LB agar plates. Spots with at least 10 distinct colonies were considered for calculation of the CFU (23).

Growth kinetics of *V. cholerae*. *Vibrio cholerae* O395N1 and the *V. cholerae* ΔoxyR strain, which contains a deletion in the *oxyR* gene encoding a hydrogen peroxide sensor protein, were transformed with plasmid pNqr1. Cells were cultivated in $\text{LB}_{7.5}$ medium, which is the standard LB medium supplemented with 50 mM potassium phosphate (pH 7.5), 100 mM Tris-Bis-Tris (pH 7.5), 50 $\mu\text{g ml}^{-1}$ streptomycin, and 200 $\mu\text{g ml}^{-1}$ ampicillin. A single colony was used to inoculate 5 ml of $\text{LB}_{7.5}$ medium, and the culture was incubated for 16 h at 37°C (180 rpm). On the next day, the cultures were normalized for their optical densities at 600 nm (by dilution in $\text{LB}_{7.5}$ medium) and used to inoculate 5 ml of freshly prepared $\text{LB}_{7.5}$ medium. To induce expression of plasmid-encoded Na^+ -NQR, the medium was supplemented with 0.1 mM, 1 mM, or 10 mM L-(+)-arabi-

nose. Cells were grown aerobically at 37°C at 180 rpm in 15-ml reaction tubes. Absorbance was measured using a Biowave CO 8000 cell density meter (WPA) until stationary phase was reached.

Dark interference contrast and fluorescence micrographs. To investigate the time course of uptake of DCFH-DA by cells, a 96-well plate with *V. cholerae* O395N1 was prepared as described. Every 2 h, aliquots of 1 μ l were placed on a poly-L-lysine-coated cover glass (Sigma-Aldrich Co. LLC.) to immobilize the cells. To avoid contamination, samples for microscopy were prepared in a laminar flow hood. To minimize the light-dependent, nonspecific formation of the fluorophore, great care was taken to avoid excessive exposure to light. The focus plane was adjusted in dark interference contrast (DIC) mode, and images were taken using a Zeiss Axio Imager AX10 microscope (AxioVision Rel.4.8 software) in fluorescent light mode using a filter set for excitation at 470 nm and emission at 525 nm. The exposure times in the DIC and fluorescence modes were held constant in all measurements (14 ms for DIC and 1,500 ms for fluorescence). The images were processed using the autocontrast function implemented in the AxioVision software.

Preparation of DCFH-DA-loaded spheroplasts. One colony of *V. cholerae* O395N1 was used to inoculate 5 ml LB_{glc} (37°C, 180 rpm). On the following day, 1 ml of this culture was used to inoculate 100 ml LB_{glc}, and growth was continued until an OD₆₀₀ of 0.6 to 0.8 was reached. Cells from 50 ml were harvested by centrifugation (GSA rotor, 7,500 rpm, 4°C). The supernatant was discarded, and the pellet was suspended in 20 ml assay buffer (45 mM Na₂SO₄, 45 μ g ml⁻¹ BSA, 18 mM Tris-H₂SO₄ [pH 7.1]). Upon addition of DCFH-DA (15 μ g ml⁻¹), cells were incubated for 3 h at 37°C to promote uptake of the dye. Spheroplast formation was achieved by the EDTA-lysozyme method initially described for *Escherichia coli* (24), modified as follows: cells from 1 ml of the suspension were harvested at 4°C using a microcentrifuge (Eppendorf 5417, 4,500 $\times$ g, 2 min) and suspended in 500 μ l of 20% (wt/vol) sucrose in 100 mM Tris-HCl (pH 8.0). After 5 min at room temperature, 10,000 units of lysozyme (from chicken egg white; Sigma-Aldrich) in 5 mM EDTA (disodium salt dihydrate), 20% (wt/vol) sucrose, and 100 mM Tris-HCl (pH 8.0) in a total volume of 250 μ l were added. Cell wall lysis was allowed to proceed for 30 min at 23°C. Note that lysis of the peptidoglycan layer in *V. cholerae* required a higher concentration of lysozyme and a prolonged incubation time compared to those used for *E. coli* as reported before (25). Images of spheroplasts in a 1:10 dilution in 20% (wt/vol) sucrose and 100 mM Tris-HCl (pH 8.0) were taken in the fluorescence mode of the Zeiss Axio Imager AX10 microscope. In a control reaction, 1 ml of the cell suspension which had been mixed with DCFH-DA was harvested and suspended in 750 μ l phosphate-buffered saline prior to fluorescence microscopy. Spheroplast formation was confirmed by phase-contrast microscopy (26, 27).

Preparation of *V. cholerae* and *E. coli* membranes for enzymatic assays. The *V. cholerae* reference strain, its variant (Δ nqr strain), and Δ nqr strain transformed with plasmids pNqr1 and pNmB were grown in LB_{glc} as described in reference 19. Expression of Na⁺-NQR and its variant lacking NqrB from plasmids was achieved by adding 6.5 mM L-(+)-arabinose to growing cultures at an OD₆₀₀ of 0.8 to 1.0. Growth was continued overnight at 30°C. Cells were harvested, washed once with 50 mM sodium phosphate (pH 8.0) and 0.5 M NaCl and stored at -80°C. Disruption of cells in a continuous lysis system (Emulsiflex C3 homogenizer; Avestin) and preparation of membranes were performed as described previously (28). The membranes were suspended in 50 mM sodium phosphate (pH 8.0) containing 0.3 M NaCl and 5% (wt/vol) glycerol to a final concentration of approximately 10 mg protein ml⁻¹ as determined by the bicinchoninic acid assay (29). They were shock frozen and stored in liquid N₂.

For production of *E. coli* membranes, *E. coli* BL21(DE3)/pLysS was streaked on an LB agar plate containing 25 μ g ml⁻¹ chloramphenicol and incubated for 16 h at 37°C. A single colony was inoculated in 5 ml of DYT medium (1.6% [wt/vol] tryptone, 1% [wt/vol] yeast extract, 0.5% [wt/vol] NaCl, 25 μ g ml⁻¹ chloramphenicol) and incubated for 16 h at 37°C (180 rpm). On the next day, 50 ml of freshly prepared DYT medium was

inoculated with 500 μ l of the culture and incubated for another 3 h (37°C, 180 rpm). An aliquot of 10 ml was used to inoculate 1 liter of DYT medium in a 5-liter Erlenmeyer flask. Cells were cultivated for 6.5 h and harvested. *E. coli* cells were processed as described above for *V. cholerae* cells to obtain membrane vesicles.

NADH oxidation and superoxide-dependent cytochrome *c* reduction activities. With the exception of membranes from *V. cholerae* Δ nqr transformed with pNqr1, which were diluted to 0.25 mg protein ml⁻¹, all membranes were adjusted to 1 mg protein ml⁻¹ with 50 mM sodium phosphate (pH 8.0), 0.3 M NaCl, 5% (wt/vol) glycerol. The oxidation of NADH (disodium salt; Sigma-Aldrich) by membranes in the presence of ubiquinone-1 (Q1) (MCAT GmbH) was accompanied by the formation of superoxide which reacted with oxidized cytochrome *c* (from bovine heart; Sigma-Aldrich) to yield reduced cytochrome *c* in equimolar amounts (30). The activity was determined in 20 mM Tris-H₂SO₄ (pH 8.0) with 50 mM Na₂SO₄, 50 μ g BSA ml⁻¹, 5% (wt/vol) glycerol, 100 μ M Q1, 40 μ M cytochrome *c*, and 100 μ M NADH. Superoxide dismutase (SOD) (bovine, recombinantly produced in *E. coli*, $\geq$ 2,500 U mg⁻¹) (Sigma-Aldrich) was added as indicated. The reaction was started by adding 10 μ l membrane suspension and monitored while the reaction mixture was stirred (25°C) in an HP 8462A diode array spectrophotometer (Hewlett Packard) at 340 nm (ϵ_{NADH} [ϵ of NADH] of 6.22 mM⁻¹) and 550 nm (ϵ_{cytred} [ϵ of reduced cytochrome *c*] of 21.5 mM⁻¹) (31).

H₂O₂ formation of *V. cholerae* and *E. coli* membranes. Rates of H₂O₂ formation by membranes from *V. cholerae* or *E. coli* were determined using the Amplex red hydrogen peroxide/peroxidase assay kit (Molecular Probes) according to the manufacturer's recommendation. A 1 $\times$ reaction solution containing 100 μ M Amplex red, 0.2 U ml⁻¹ horseradish peroxidase (HRP) in 50 mM sodium phosphate (pH 7.4) was prepared. The membranes were diluted to a concentration of 0.01 mg ml⁻¹ (with the exception of membranes from *V. cholerae* Δ nqr/pNqr1 which were diluted to 0.005 mg ml⁻¹). A typical reaction mixture contained 50 μ l of the 1 $\times$ reaction solution, 10 μ l of diluted membranes, and 30 μ l of a 3 $\times$ reaction buffer (66 mM Tris-H₂SO₄ [pH 8.0], 165 mM Na₂SO₄, 165 μ g ml⁻¹ BSA, 16.5% glycerol, 330 μ M Q1). The reaction was started with 10 μ l of NADH (1 mM in H₂O) and recorded over a period of 30 min at 25°C. The formation of resorufin was monitored in a Tecan Infinite F200 Pro plate reader using the i-control software. Specific activity was determined using the extinction coefficient for resorufin at 560 nm (ϵ_{560} = 58,000 cm⁻¹ M⁻¹) (32).

RESULTS

Uptake of DCFH-DA and conversion to DCF by *V. cholerae*.

DCFH-DA is readily oxidized in solution by nonspecific reactions, which are not related to ROS (33). Thus, one needs to consider that an increase in DCF fluorescence of a suspension of *V. cholerae* cells does not exclusively result from the increase of intracellular superoxide. We first addressed whether *V. cholerae* cells take up DCFH-DA in sufficient amounts and whether cytosolic esterases convert DCFH-DA to DCFH, followed by conversion to DCF. The *V. cholerae* reference strain was incubated in the presence of 15 μ g ml⁻¹ DCFH-DA, and the time course of DCFH-DA uptake monitored from the formation of the fluorophore DCF was monitored by fluorescence microscopy. After 2 h, all cells inspected in the DIC mode also showed a strong fluorescence signal (Fig. 1A). The intensities of the fluorescence signals of individual cells were very similar, indicating that cells had taken up similar amounts of the fluorophore. There was no specific cellular localization, indicating an equal distribution of DCF in the cell. After 6 h, the intensity of fluorescence of cells increased (Fig. 1B), demonstrating that the amount of DCF formed was correlated with incubation time. This indicated that formation of the fluorescent DCF resulted from metabolic activity of *V. cholerae* cells and suggested respiration-

FIG 1 Uptake of DCFH-DA and conversion to DCF by *Vibrio cholerae*. *V. cholerae* cells were treated with $15 \mu\text{g ml}^{-1}$ DCFH-DA. After 2 h (A) and after 6 h (B), $1 \mu\text{l}$ of the cell suspension was applied onto a lysine-coated glass slide to immobilize the cells, and microscopic pictures in the DIC (top panels) and fluorescence mode (bottom panels) were taken. Bar, $10 \mu\text{m}$.

dependent formation of superoxide. The strong fluorescence of cells suggested that DCF was localized inside the cell, but since *V. cholerae* is a Gram-negative bacterium, a periplasmic localization of the fluorophore was also considered. If the fluorophore were predominantly accumulated in the periplasm surrounding the cell, formation of DCF fluorescence would reflect extracellular formation of superoxide (30). To address this question, *V. cholerae* cells loaded with DCFH-DA were treated with lysozyme which results in the formation of spheroplasts due to lysis of the peptidoglycan layer. The inner membrane stays intact, giving rise to smaller and round spheroplasts which can be easily distinguished from the larger, comma-shaped, intact bacteria (Fig. 2). *V. cholerae* spheroplasts and *V. cholerae* cells both exhibit strong fluorescence, indicating that DCFH-DA was taken up by *V. cholerae* cells and converted to fluorescent DCF in the cytoplasm. Comparable results were obtained in two independent experiments.

Critical assessment of the method for fluorescence-based detection of intracellular ROS. Several parameters could affect the outcome of the experiment. (i) Reactants other than ROS might lead to the activation of DCFH-DA and its subsequent oxidation, resulting in nonspecific DCF fluorescence. To address this problem, fluorescence emission of DCFH-DA dissolved in different assay buffers was recorded. (ii) Photoreduction of DCFH-DA, i.e., the light-induced activation of DCFH-DA, is known to drastically diminish the signal-to-noise ratio (34), so we varied the exposure time. (iii) The emission of a fluorophore is very sensitive to O_2 partial pressure, since O_2 is an efficient quencher of fluorescence (35, 36). We confirmed this for DCFH-DA/DCF (see Fig. S1 in the supplemental material); therefore, aerobic experiments were performed in small reaction mixture volumes to ensure saturation

FIG 2 Cytoplasmic localization of fluorescent DCF. *V. cholerae* cells treated with DCFH-DA for 4 h were inspected under a fluorescence microscope in the DIC mode or in the fluorescence mode. (Left) Whole cells after resuspension in PBS; (right) *V. cholerae* spheroplasts from the same culture after 30 min treatment with lysozyme. Bar, $10 \mu\text{m}$.

with O_2 . To address the influence of reactants, we compared the fluorescence of DCFH-DA conversion products in the absence of cells in a Tris-based buffer with moderate salinity, in salt-based minimal medium with glucose, and in LB (Fig. 3). In LB medium which contains a large amount of organic compounds, fluorescence reached $\sim 480,000$ relative fluorescence units (RFU), which corresponds to an approximately 10- to 50-fold increase compared to fluorescence in Tris buffer or in salt-based minimal me-

FIG 3 Effect of medium composition on conversion of DCFH-DA to a fluorescent compound. Specifically, $15 \mu\text{g ml}^{-1}$ DCFH-DA, $10 \mu\text{l}$ of 180 mM Tris- H_2SO_4 (pH 7.1), 450 mM Na_2SO_4 , and 0.45 mg ml^{-1} BSA were mixed with water (circles), salt-based minimal medium with glucose (squares), or LB medium (triangles). During incubation at 37°C , the emission at 535 nm was monitored at the indicated time points over a period of 24 h. Measurements were performed in a 96-well plate. Values are means $\pm$ standard deviations (SD) (error bars) from 8 replicate experiments.

FIG 4 Light-dependent increase in fluorescence emission due to DCFH-DA conversion. Images from *V. cholerae* cells that were incubated with $15 \mu\text{g ml}^{-1}$ DCFH-DA for 4 h in the dark were taken after different times of exposure to the excitation wavelength. The images were taken immediately after preparation (A), after 2 min of light exposure (B), and after 5 min of light exposure (C). Bars, $10 \mu\text{m}$.

dium plus glucose (Fig. 3). In minimal medium with glucose, a fluorescence intensity of $\sim 51,000$ RFU was reached after 24 h, which is about fivefold higher than the intensity observed in Tris-based buffer ($\sim 9,500$ RFU). The Tris-based buffer had the smallest effect with respect to nonspecific conversion of DCFH-DA and was used in the following experiments. To assess the contribution of photoreduction of DCFH-DA, we took images of DCFH-DA-loaded *V. cholerae* cells with the fluorescence microscope after different exposure times to the exciting light (Fig. 4). In fluoromicrographs taken without previous illumination, the cells, but not the background, exhibited DCF fluorescence. Two minutes of light exposure resulted in increased fluorescence of both cells and background, and after 5 min of illumination, background fluorescence represented the major contribution to overall fluorescence. Therefore, the nonspecific formation of oxygen radicals due to photoreduction of DCFH-DA during excitation of the fluorophore is substantial and must be taken into account when recording the biological conversion to DCF to estimate ROS formation in bacterial cells. The duration of exposure to the exciting light and the frequency of fluorescence measurements should be kept as low as possible in order to diminish background fluorescence.

DCFH-DA is not toxic for *V. cholerae*. In cell suspensions of identical optical densities which had been incubated with the dye for 16 h, the CFU of the *V. cholerae* reference strain was 1.1×10^8 compared to 1.2×10^8 in the absence of the dye. It is concluded that DCFH-DA or its conversion products are not toxic for *V. cholerae*. We compared the viability of the different *V. cholerae* strains used in this study and determined the CFU before and after 16 h of incubation in the presence of DCFH-DA (Table 1). Only a very moderate decline in CFU numbers was observed, and it was not influenced by the genetic background of the strains. Note that the decrease in CFU does not necessarily reflect a decrease in the number of viable cells. *V. cholerae* may enter a viable but nonculturable state (VBNC) of survival. In the VBNC state, *V. cholerae*

maintains metabolic activity but cannot be grown on solid media (37, 38).

Enhanced DCF formation in the presence of respiring *V. cholerae*. We monitored the increase in DCF fluorescence in the presence and absence of cells from the *V. cholerae* reference strain in the Tris-based assay buffer and demonstrated superoxide-dependent transformation of DCFH-DA (Fig. 5). In the absence of cells, the fluorescence signal at 535 nm increased due to nonspecific reactions, and this increase in intensity was similar to the increase observed in the presence of cells during the first 5 h of incubation. After 6 h, the fluorescence intensity was somewhat stronger in the presence of *V. cholerae* cells compared to the cell-free control, indicating superoxide-dependent conversion of the dye in the course of cellular respiration. From 6 h to 24 h, DCF formation strongly increased in the presence of cells. Fluorescence of the cell-free control reached a plateau after 20 h. At this time, *V. cholerae* cells continued to catalyze the formation of DCF, reaching 1.8-fold-higher fluorescence intensity compared to that of the control after 24 h (Fig. 5). It is noteworthy that the ratio of DCF fluorescence monitored in the presence of respiring cells and in the cell-free control is subject to variation because of the self-propagating redox cycling reactions (33). Therefore, cell-free controls must always be included in the experiments. We observed a significant increase of DCF fluorescence with cells compared to the cell-free controls in more than 100 experiments.

Respiratory and ROS formation activities of *Vibrio cholerae* membranes. We next addressed the source for reactive oxygen

TABLE 1 Survival of *V. cholerae* strains during fluorescence measurements^a

Strain	CFU	
	t_0	t_1 (16 h)
<i>V. cholerae</i> O395N1	9.0×10^8	1.1×10^8
<i>V. cholerae</i> Δnqr	1.6×10^9	1.1×10^8
<i>V. cholerae</i> $\Delta nqr/pNqr1$	1.5×10^9	5.0×10^8
<i>V. cholerae</i> $\Delta nqr/pNmB$	1.6×10^9	1.4×10^8

^a The CFU were determined immediately after mixing the cells with assay buffer containing DCFH-DA (time zero [t_0]) and after 16 h of incubation (time 1 [t_1]) at 37°C in 96-well plates in an orbital shaker.

FIG 5 *V. cholerae* cells promote conversion of DCFH-DA to fluorescent DCF. The fluorescence of $15 \mu\text{g ml}^{-1}$ DCFH-DA in 18 mM Tris- H_2SO_4 (pH 7.1), 45 mM Na_2SO_4 , and 0.045 mg ml^{-1} BSA was monitored at 535 nm during incubation at 37°C . Measurements were performed in a 96-well plate. Mean values $\pm$ SD from eight replicate experiments are presented. Symbols: circles, no cells added; squares, with cells of the *V. cholerae* reference strain.

TABLE 2 Comparison of NADH oxidation and ROS formation activities of membranes from different *V. cholerae* strains with membranes from *E. coli*

Strain	Sp act ($\mu\text{mol min}^{-1} \text{mg}^{-1}$) ^a				
	No SOD		150 U SOD		Resorufin formation
	NADH oxidation	Cytochrome <i>c</i> reduction	NADH oxidation	Cytochrome <i>c</i> reduction	
<i>V. cholerae</i> O395N1	6.3 $\pm$ 0.5	9.8 $\pm$ 0.6	4.9 $\pm$ 0.4	1.8 $\pm$ 0.2	0.104 $\pm$ 0.004
<i>V. cholerae</i> Δnqr	0.29 $\pm$ 0.1	0.18 $\pm$ 0.01	0.20 $\pm$ 0.02	0.07 $\pm$ 0.01	0.026 $\pm$ 0.003
<i>V. cholerae</i> $\Delta nqr/pNqr1$	23.2 $\pm$ 1.8	42.6 $\pm$ 2.8	21.7 $\pm$ 2.0	6.9 $\pm$ 0.4	0.183 $\pm$ 0.013
<i>V. cholerae</i> $\Delta nqr/pNmB$	2.2 $\pm$ 0.2	5.1 $\pm$ 0.3	1.6 $\pm$ 0.1	2.9 $\pm$ 0.1	0.077 $\pm$ 0.004
<i>E. coli</i> BL21(DE3)/pLysS	1.4 $\pm$ 0.02	0.13 $\pm$ 0.01	1.4 $\pm$ 0.1	0.03 $\pm$ 0.01	0.026 $\pm$ 0.003

^a Values are means $\pm$ standard deviations (SD) from three replicate experiments.

species formed in the cytoplasm of *V. cholerae*, assuming that the respiratory enzymes played an important role in this process. *V. cholerae* operates a redox-driven Na^+ pump in the NADH:quinone segment of its respiratory chain, but it does not possess a NADH dehydrogenase belonging to the complex I family of enzymes (2). The Na^+ -translocating NADH:quinone oxidoreductase produced superoxide *in vitro* (8) and participated in the extracellular formation of superoxide by *V. cholerae* *in vivo* (30). We compared the ROS formation activities of membranes from different *V. cholerae* strains with various amounts of active NQR. The amount of H_2O_2 and $\text{O}_2^{\cdot-}$ released by Na^+ -NQR during NADH turnover was determined using Amplex red/HRP or cytochrome *c*, respectively. Oxidized cytochrome *c* is an artificial electron acceptor for $\text{O}_2^{\cdot-}$, which undergoes one-electron reduction to yield reduced cytochrome *c* (cytc_{red}) and O_2 in a stoichiometric manner (39). Membranes from the *V. cholerae* reference strain with endogenous amounts of Na^+ -NQR were compared with membranes from the Δnqr variant lacking the Na^+ -NQR and with membranes from Δnqr strains transformed with plasmid pNqr1 coding for the holo-NQR complex or with plasmid pNmB coding for a subcomplex lacking the NqrB subunit. For a control, we investigated NADH-dependent cytochrome *c* reduction by *E. coli* membranes which do not contain Na^+ -NQR (40). In *E. coli*, NADH is oxidized by NADH dehydrogenase I (NDH-I) (complex I) and NDH-II, the alternative NADH dehydrogenase (40). NDH-II is also present in *V. cholerae* and catalyzes NADH oxidation in strains lacking a functional Na^+ -NQR (30). The rates of reduction of cytochrome *c* reflected the amounts of active Na^+ -NQR in the membranes. Membranes from the *V. cholerae* reference strain exhibited a rate of $9.8 \pm 0.6 \mu\text{mol cytc}_{\text{red}} \text{min}^{-1} \text{mg}^{-1}$, in marked contrast to membranes from *V. cholerae* Δnqr ($0.18 \pm 0.01 \mu\text{mol cytc}_{\text{red}} \text{min}^{-1} \text{mg}^{-1}$) or *E. coli* ($0.13 \pm 0.01 \mu\text{mol cytc}_{\text{red}} \text{min}^{-1} \text{mg}^{-1}$) which do not contain Na^+ -NQR. The superoxide formation activities of membranes from *V. cholerae* $\Delta nqr/pNqr1$ which are highly enriched in active Na^+ -NQR were 4- to 5-fold higher ($42.6 \pm 2.8 \mu\text{mol min}^{-1} \text{mg}^{-1}$ in) than that of the reference strain. We observed a significant cytochrome *c* reduction activity ($5.1 \pm 0.3 \mu\text{mol min}^{-1} \text{mg}^{-1}$) in *V. cholerae* $\Delta nqr/pNmB$, indicating that Na^+ -NQR lacking the NqrB subunit produces superoxide, albeit at a rate lower than that of intact Na^+ -NQR. Importantly, Na^+ -NQR lacking the membrane-bound NqrB subunit still exhibited NADH oxidation activity, catalyzed by the NqrF subunit (Table 2). Electrons for cytochrome *c* reduction were mostly derived from $\text{O}_2^{\cdot-}$, since the reaction was strongly inhibited by superoxide dismutase (SOD) (Table 2). With membranes

from the overproducing *V. cholerae* strain containing the largest amount of active Na^+ -NQR, rates of one-electron cytochrome *c* reduction were almost twofold higher than the rates of oxidation of NADH, a donor of two electron equivalents. This is in accord with a net stoichiometry of one NADH molecule oxidized per two cytochrome *c* molecules reduced under the given assay conditions. Even with membranes from the reference strain, approximately 80% of the electron equivalents obtained from NADH went into cytochrome *c* reduction. NADH-dependent cytochrome *c* reduction activities of membranes from *V. cholerae* Δnqr or *E. coli* were significantly lower but also decreased in the presence of SOD, indicating superoxide formation by other respiratory enzymes. Possible sources for ROS are the alternative NADH dehydrogenase NDH-II (41, 42) present in *V. cholerae* and *E. coli* (2, 43) or the cytochrome *c* reductase (44).

Next, we investigated the formation of hydrogen peroxide by Na^+ -NQR using the Amplex red/HRP method. In the presence of HRP and H_2O_2 , resorufin is formed (from Amplex red) in a 1:1 stoichiometry (45). As observed with $\text{O}_2^{\cdot-}$, the amount of released H_2O_2 correlated with the amount of active Na^+ -NQR in the membranes: $0.104 \pm 0.004 \mu\text{mol H}_2\text{O}_2 \text{min}^{-1} \text{mg}^{-1}$ with the reference strain, $0.183 \pm 0.013 \mu\text{mol H}_2\text{O}_2 \text{min}^{-1} \text{mg}^{-1}$ with *V. cholerae* $\Delta nqr/pNqr1$, and $0.077 \pm 0.004 \mu\text{mol H}_2\text{O}_2 \text{min}^{-1} \text{mg}^{-1}$ with *V. cholerae* $\Delta nqr/pNmB$. Membranes from *V. cholerae* Δnqr and from *E. coli* lacking Na^+ -NQR exhibited a low H_2O_2 formation rate of $0.026 \pm 0.003 \mu\text{mol H}_2\text{O}_2 \text{min}^{-1} \text{mg}^{-1}$. Note that the rates of cytochrome *c* formation by *V. cholerae* membranes were 2 orders of magnitude higher than the H_2O_2 formation rates, indicating that Na^+ -NQR predominantly forms $\text{O}_2^{\cdot-}$. The rather low rates of H_2O_2 formation could also result from nonenzymatic dismutation of superoxide (46).

Na^+ -NQR contributes to the formation of intracellular superoxide in *V. cholerae*. We compared the fluorescence of different *V. cholerae* strains to test whether the signal was dependent on the amount of active Na^+ -NQR (Fig. 6). In Fig. 6A, the fluorescence of cell suspensions from *V. cholerae* strains expressing different Na^+ -NQR variants after 16 h relative to the cell-free controls is presented. Figure 6B shows the cell densities of the suspensions after 16 h (OD_{595}) which were determined in parallel using the absorbance mode of the plate reader. Note that the fluorescence intensities shown in Fig. 6A are not corrected for cell densities shown in Fig. 6B, since there was little variation in the latter. *V. cholerae* strains with no Na^+ -NQR (Δnqr), endogenous amounts of active Na^+ -NQR (wild type [wt]), large amounts of active Na^+ -NQR ($\Delta nqr/pNqr1$), or large amounts of a truncated

FIG 6 Na⁺-NQR as the source for intracellular superoxide in *V. cholerae*. The *V. cholerae* reference strain (wild type [wt]), its variant lacking the Na⁺-NQR (Δnqr strain), and the Δnqr strain transformed with pNqr1 coding for active NQR or with pNmB coding for a subcomplex devoid of the NqrB subunit were grown in LB_{glc} in the presence (+) or absence (–) of arabinose, which induces expression of Na⁺-NQR variants in plasmid-carrying strains. Superoxide-dependent DCF formation by cells was monitored in assay buffer without nutrients. Fluorescence signals of cell suspensions were analyzed after 16 h using the i-control software and compared to the fluorescence signal of a cell-free control. Simultaneously, the absorbance at 595 nm was recorded. Mean values plus SD are from three biological replicate samples, each analyzed with three technical replicate samples. Values that are significantly different ($P = 0.045$ by the Student *t* test) are indicated by the bar and asterisk ($n = 9$).

variant of the Na⁺-NQR lacking subunit NqrB (Δnqr /pNmB) were analyzed for DCF fluorescence emission. To induce expression of Na⁺-NQR variants, arabinose was added to the medium during growth of cells prior to their analysis. The *V. cholerae* reference strain (wt) and its variant lacking the Na⁺-NQR (Δnqr) were also grown with or without arabinose added to exclude an indirect effect of this carbon source on superoxide formation. The *V. cholerae* reference strain exhibited a significant increase in cytoplasmic superoxide formation compared to the *nqr* deletion strain. In the absence of arabinose, e.g., after growth on mixed amino acids without added sugar, the relative increase in fluores-

cence was 1.66 ± 0.1 -fold compared to 1.26 ± 0.1 -fold in the *nqr* deletion strain. It is concluded that Na⁺-NQR represents a source for cytoplasmic ROS in *V. cholerae*. This was further supported by monitoring ROS formation in the presence of HQNO, a specific inhibitor of Na⁺-NQR (22). For the *V. cholerae* reference strain, the fluorescence intensities of $751,282 \pm 31,629$ RCF observed in the absence of the inhibitor decreased to $413,267 \pm 53,612$ RCF in the presence of $5 \mu\text{M}$ HQNO. In contrast, the *nqr* deletion strain displayed comparable fluorescence intensities, irrespective of the presence ($218,817 \pm 8,159$ RCF) or absence ($201,983 \pm 11,541$ RCF) of the inhibitor. The mean values plus standard deviations (SD) are from nine experiments, respectively, after 15 h of incubation with DCFH-DA.

With arabinose added, the increase in DCF fluorescence was less pronounced, with 1.18 ± 0.1 -fold in the reference strain compared to 1.12 ± 0.06 -fold in the Δnqr strain (Fig. 6). The highest fluorescence intensity (2.2 ± 0.3 -fold) was observed with the *nqr* deletion strain transformed with plasmid pNqr1 coding for active Na⁺-NQR after growth in the presence of arabinose, leading to strong overproduction of active Na⁺-NQR containing the six subunits (NqrACDEF). Overproduction of the NqrACDEF variant of the complex lacking the large, membrane-bound NqrB subunit did not result in a significant increase in relative fluorescence compared to the *nqr* deletion strain after 16 h. Yet, membranes from this strain exhibited substantial superoxide formation activity (Table 2), and after a total incubation time of 24 h, the relative increase in fluorescence emission in the Δnqr /pNmB strain and the *nqr* deletion strain were 1.18 ± 0.6 -fold and 1.1 ± 0.06 -fold, respectively, with a *P* value of 0.00118 ($n = 9$). This demonstrated enhanced formation of cytoplasmic superoxide in cells expressing the NqrACDEF complex compared to the *nqr* deletion strain.

V. cholerae $\Delta oxyR$ is hypersensitive to overexpression of Na⁺-NQR. OxyR is a transcriptional regulator that is activated by H₂O₂ to promote the synthesis of ROS protection enzymes like catalase under conditions of oxidative stress (47, 48). We showed that the Na⁺-NQR is a source for O₂^{•−} and H₂O₂ and reasoned that *V. cholerae* critically depended on its ROS protection enzymes, especially under conditions of overexpression of Na⁺-NQR, which leads to increased ROS formation (Table 2). To test this hypothesis, a *V. cholerae* *oxyR* deletion mutant was constructed and transformed with pNqr1. We monitored the growth of *V. cholerae* $\Delta oxyR$ /pNqr1 at increasing arabinose concentrations to vary the expression level of Na⁺-NQR and, concomitantly, the amount of ROS formed in cells. Growth of the *V. cholerae* reference strain transformed with pNqr1 was monitored in parallel (Fig. 7). With no arabinose added, both strains exhibited the same growth phenotype and reached a final OD₆₀₀ of 1.35 ± 0.05 (reference strain) and 1.34 ± 0.1 ($\Delta oxyR$ /pNqr1) in the stationary phase after 24 h (Fig. 7). The addition of 0.1 mM arabinose to the growth medium resulted in a slight increase in the final cell yield with the reference strain (Fig. 7), perhaps caused by an increase in membrane potential generated by the Na⁺-NQR (49). Increasing the arabinose concentration to 1 mM and 10 mM did not affect the reference strain but led to a decrease in final cell densities of *V. cholerae* $\Delta oxyR$ /pNqr1 by 11% (1 mM arabinose) and 26% (10 mM arabinose) compared to *V. cholerae*/pNqr1 (Fig. 7). The *oxyR* deletion strain, but not the *V. cholerae* reference strain, was sensitive to overexpression of Na⁺-NQR. We conclude

FIG 7 *Vibrio cholerae* $\Delta oxyR$ is hypersensitive to overexpression of Na^+ -NQR. Growth of *V. cholerae*/pNqr1 and *V. cholerae* $\Delta oxyR$ /pNqr1 was monitored in LB medium containing 50 mM potassium phosphate (pH 7.5) and 100 mM Tris-Bis-Tris (pH 7.5). To vary the expression level of Na^+ -NQR, the medium contained 0 mM, 0.1 mM, 1 mM, or 10 mM arabinose. Absorbance at 600 nm is shown in arbitrary units (AU). Symbols: closed circles, *V. cholerae*/pNqr1; open circles, *V. cholerae* $\Delta oxyR$ /pNqr1.

that ROS formed by the Na^+ -NQR are detoxified in the *V. cholerae* reference strain but are harmful to the *oxyR* deletion strain.

DISCUSSION

We describe a method for detection of oxygen radicals formed in the cytoplasm of *V. cholerae*. The major source of cytoplasmic reactive oxygen species is Na^+ -NQR. As observed with other respiratory enzymes, the sources of ROS are flavins, which (in their reduced form) react with oxygen, before the electrons are transferred to a one-electron acceptor in electron transfer distance to the flavin (50). The rates at which oxygen radicals are formed depend on the properties of the flavin binding site (especially the solvent accessibility of the isoalloxazine ring) and can vary over several orders of magnitude in individual enzymes (51). Flavoproteins are classified by their tendency to form either $O_2^{\cdot-}$ or H_2O_2 as predominant reactive oxygen species (50, 52). Na^+ -NQR predominantly produces superoxide at rates of $9.8 \pm 0.6 \mu\text{mol min}^{-1}$ per mg total protein in membrane vesicles, while H_2O_2 formation rates are 2 orders of magnitude lower. This suggests that H_2O_2 is formed indirectly by the nonenzymatic dismutation of $O_2^{\cdot-}$. Similar product patterns were observed with succinate dehydrogenase which belongs to the class of superoxide-forming flavoenzymes (51). We could identify the site for cytoplasmic superoxide formation in Na^+ -NQR. This respiratory Na^+ pump contains four flavins, which represent putative electron donors for O_2 (Fig. 8). The two iron centers are not considered sites of superoxide formation, since they are buried within the NqrF subunit or in the interface between NqrD and NqrE subunits, and therefore are not readily approached by oxygen. The midpoint potentials of the flavin cofactors in Na^+ -NQR were determined by Bogachev et al. (53). For the FAD/FADH₂ pair in NqrF, the midpoint potential is -200 mV. For FMN_C in Na^+ -NQR, midpoint potential values of -250 mV for the $FlH^- \rightleftharpoons Fl^-$ transition and of -200 mV for the $Fl^- \rightleftharpoons Fl$ transition were determined. The redox transitions of FAD or

FMN_C operate at potentials which are lower than the midpoint potential for the superoxide/oxygen couple with -160 mV (54), making FAD and FMN_C putative electron donors for O_2 . In the same study by Bogachev and coworkers, the midpoint potentials

FIG 8 Reduced or semireduced flavins in NqrF and NqrC are sites for superoxide formation in Na^+ -NQR. The six subunits of Na^+ -NQR (NqrABCDEF) located in the inner membrane of *V. cholerae* (gray bar) are depicted with their associated cofactors. Electron transfer occurs from flavin adenine dinucleotide (FAD) (in NqrF) via two iron clusters (NqrF and NqrD/E) to the periplasmic localized FMN_C (flavin mononucleotide in NqrC). From here, electrons are transferred to the FMN_B (NqrB) and a riboflavin (RF) located in the same subunit until the electrons are finally transferred to ubiquinone 8 (Q8). The localization of the Q binding site is controversial (NqrA or NqrB*).

for FMN_B and riboflavin, both on NqrB, were determined to be −130 mV and −10 mV, respectively (53), making a direct electron transfer from flavins in NqrB to oxygen thermodynamically unfavorable. The FMN_C of NqrC is located in the periplasm (3), and therefore, O₂^{•−} formation would lead to extracellular and not intracellular formation of superoxide (Fig. 8). Functional similarities of the electron input modules of Na⁺-NQR (subunit NqrF) and complex I imply a similar flavin site mechanism for superoxide production in Na⁺-NQR and complex I. While complex I harbors a noncovalently FMN as a mediator for electron transport between NADH and the [2Fe-2S] cluster, Na⁺-NQR contains an FAD for NADH oxidation, with a [2Fe-2S] center in close proximity (3). Thus, as reported for complex I and aldehyde oxidase (55), one- and two-electron-reduced FAD may act as an electron donor for superoxide formation in the Na⁺-NQR. In this case, cytoplasmic superoxide formation would mainly depend on NADH oxidation activity catalyzed by FAD in the cytoplasmic domain of subunit NqrF (Fig. 8). We further corroborated this hypothesis by analyzing *in vitro* NADH oxidation and superoxide/hydrogen peroxide formation and cytoplasmic ROS formation *in vivo*, with *V. cholerae* Δ*nqr* transformed with a plasmid encoding Na⁺-NQR lacking the large, membrane-bound NqrB subunit with its two flavins, one flavin (a riboflavin) exposed to the cytoplasm and the other flavin (a covalently bound FMN) exposed to the periplasm (Fig. 8). Although NADH and superoxide formation activity determined *in vitro* with membranes was decreased compared to wild-type Na⁺-NQR, cells producing the NqrACDEF variant still exhibited cytoplasmic superoxide formation *in vivo* to levels which were higher than the levels observed with the *nqr* deletion strain. This is in accord with the notion of *in vivo* superoxide formation by FAD on NqrF (Fig. 8). In a previous study (30), we identified the Na⁺-NQR as a source for extracellular superoxide formed during respiration of *V. cholerae*. The extracellular superoxide production was attributed to one-electron-reduced ubisemiquinone formed by Na⁺-NQR, since ubisemiquinone species are known sources for superoxide in respiratory enzymes (56). At that time, the periplasmic flavin (FMN_C) which was recently revealed in the 3D structure of the Na⁺-NQR (3) was not considered. The formation of extracellular superoxide by the periplasmic FMN_C of Na⁺-NQR therefore needs to be addressed in future studies (Fig. 8).

Besides its role in respiration, Na⁺-NQR is the focus of research for its effect on virulence. NQR is found in many other pathogens, including *Vibrio vulnificus*, *Neisseria gonorrhoeae*, *Haemophilus influenzae*, *Yersinia pestis*, and *Pseudomonas aeruginosa* (4). In *V. cholerae*, Na⁺-NQR affects expression of *toxT*, a transcriptional activator which controls expression of virulence genes (57, 58). Later, *nqrA* and *nqrF* were identified to be crucial for colonization of the small intestine in mice (59), which was attributed to a diminished acid tolerance response (ATR) of *V. cholerae* devoid of functional NQR, caused by impaired sodium ion homeostasis (59). Our results suggest another link between Na⁺-NQR activity and ATR response. In *V. vulnificus*, an inducible MnSOD is crucial for ATR (60) and increases virulence (61). The expression of MnSOD is regulated via the *soxRS* regulon and is stimulated in response to O₂^{•−} (60, 62). Superoxide produced by Na⁺-NQRs present in *Vibrio* sp. is likely to contribute to this activation of ATR, hereby enhancing virulence in the host.

ACKNOWLEDGMENT

V.M. thanks the Landesgraduiertenförderung (Baden-Württemberg) for financial support.

FUNDING INFORMATION

This work, including the efforts of Valentin Muras, was funded by Land Baden-Württemberg (scholarship).

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