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NqrM (DUF539) Protein Is Required for Maturation of Bacterial Na^+-Translocating NADH:Quinone Oxidoreductase

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ABSTRACT

Na^+-translocating NADH:quinone oxidoreductase (Na^+-NQR) catalyzes electron transfer from NADH to ubiquinone in the bacterial respiratory chain, coupled with Na^+ translocation across the membrane. Na^+-NQR maturation involves covalent attachment of flavin mononucleotide (FMN) residues, catalyzed by flavin transferase encoded by the *nqr*-associated *apbE* gene. Analysis of complete bacterial genomes has revealed another putative gene (*duf539*, here renamed *nqrM*) that usually follows the *apbE* gene and is present only in Na^+-NQR-containing bacteria. Expression of the *Vibrio harveyi nqr* operon alone or with the associated *apbE* gene in *Escherichia coli*, which lacks its own Na^+-NQR, resulted in an enzyme incapable of Na^+-dependent NADH or reduced nicotinamide hypoxanthine dinucleotide (dNADH) oxidation. However, fully functional Na^+-NQR was restored when these genes were coexpressed with the *V. harveyi nqrM* gene. Furthermore, *nqrM* lesions in *Klebsiella pneumoniae* and *V. harveyi* prevented production of functional Na^+-NQR, which could be recovered by an *nqrM*-containing plasmid. The Na^+-NQR complex isolated from the *nqrM*-deficient strain of *V. harveyi* lacks several subunits, indicating that *nqrM* is necessary for Na^+-NQR assembly. The protein product of the *nqrM* gene, NqrM, contains a single putative transmembrane α-helix and four conserved Cys residues. Mutating one of these residues (Cys33 in *V. harveyi* NqrM) to Ser completely prevented Na^+-NQR maturation, whereas mutating any other Cys residue only decreased the yield of the mature protein. These findings identify NqrM as the second specific maturation factor of Na^+-NQR in proteobacteria, which is presumably involved in the delivery of Fe to form the $(Cys)_4$[Fe] center between subunits NqrD and NqrE.

IMPORTANCE

Na^+-translocating NADH:quinone oxidoreductase complex (Na^+-NQR) is a unique primary Na^+ pump believed to enhance the vitality of many bacteria, including important pathogens such as *Vibrio cholerae*, *Vibrio parahaemolyticus*, *Haemophilus influenzae*, *Neisseria gonorrhoeae*, *Pasteurella multocida*, *Porphyromonas gingivalis*, *Enterobacter aerogenes*, and *Yersinia pestis*. Production of Na^+-NQR in bacteria requires Na^+-NQR-specific maturation factors. We earlier identified one such factor (ApbE) that covalently attaches flavin residues to Na^+-NQR. Here we identify the other protein factor, designated NqrM, and show that NqrM and ApbE suffice to produce functional Na^+-NQR from the *Vibrio harveyi nqr* operon. NqrM may be involved in Fe delivery to a unique Cys_4[Fe] center during Na^+-NQR assembly. Besides highlighting Na^+-NQR biogenesis, these findings suggest a novel drug target to combat Na^+-NQR-containing bacteria.

Na^+-translocating NADH:quinone oxidoreductase (Na^+-NQR) is a redox-driven sodium pump operating in the respiratory chains of various bacteria, including several pathogens (1). This enzyme is a functional analog of H^+-translocating NADH:quinone oxidoreductase, known as mitochondrial complex I or bacterial NDH-1. Bacteria may also contain NDH-2, an NADH:quinone oxidoreductase that is not involved in energy transduction. The three bacterial enzymes are structurally very different: NDH-1 is formed by 13 to 14 and Na^+-NQR by six dissimilar subunits, whereas NDH-2 is a monomeric enzyme (2). The enzymes are found in different combinations in different aerobic bacteria, but at least one is commonly present.

Na^+-NQR catalyzes electron transfer from NADH to ubiquinone in the respiratory chain, coupled with Na^+ translocation across the membrane. *In vitro*, the enzyme exhibits Na^+-dependent quinone reductase activity and Na^+-independent NADH dehydrogenase activity with soluble quinone (e.g., menadione) as an electron acceptor (3). Only the flavin adenine dinucleotide (FAD)-binding domain of the NqrF subunit is apparently required for the latter activity (4), which does not depend on Na^+ and is resistant to low 2-heptyl-4-hydroxyquinoline N-oxide (HQNO) concentrations (5). In contrast, quinone reductase activity is manifested only by a complete Na^+-NQR complex and is Na^+ dependent and HQNO sensitive (5, 6). Both NADH and reduced nicotinamide hypoxanthine dinucleotide (dNADH) can be used as substrates in these reactions (6, 7).

Six subunits of Na^+-NQR (NqrA to -F) (8) with unique amino acid sequences are encoded by six genes of the *nqr* operon (9, 10). The enzyme contains a [2Fe-2S] cluster and FAD in subunit NqrF (4, 11), riboflavin between subunits NqrB and NqrE (12, 13), an

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TABLE 1 Bacterial strains and plasmids used in this study

Name	Relevant details	Reference or source
Strains		
V. harveyi		
R3	ATCC 33843; Rf^r	18
VHtag60	R3; C-terminal 6×His tag fusion in *nqrF*; Km^r Rf^r	22
VHDM15	VHtag60; *nqrM*$_1$::Cm^r Km^r Rf^r Cm^r	This work
VHDDM109	VHtag60; *nqrM*$_1$::Cm^r *nqrM*$_2$::Em^r Km^r Rf^r Cm^r Em^r	This work
V. cholerae O395N1 *toxT*::*lacZ* Δ*nqrABCDEF*	Δ*nqr* Sm^r	28
K. pneumoniae		
NTUH-K2044	Wild type	41
KNU210	NTUH-K2044; *nuoB*::Cm^r Rf^r Cm^r	29
DUF539	NTUH-K2044; *nuoB*::Cm^r *nqrM*::Km^r Rf^r Cm^r Km^r	This work
E. coli		
ANN091	F^- *thi nuoI*::Km^r Sm^r Km^r	26
Sm10 λpir	*thi thr leu tonA lacY supE recA*::RP4-2-Tc::Mu Km^r	42
Plasmids		
pNQ	pBAD-TOPO bearing the *V. harveyi nqr* operon; Ap^r	This work
pNQ_AE	pBAD-TOPO bearing the *V. harveyi nqr* operon and *apbE* gene; pUC *ori* Ap^r	This work
pNQ_AE_NqrM	pBAD-TOPO bearing the *V. harveyi nqr* operon, *apbE* gene, and *nqrM*$_1$ gene; Ap^r	This work
pNQ_NqrM	pBAD-TOPO bearing the *V. harveyi nqr* operon and *nqrM*$_1$ gene; Ap^r	This work
pBAD_NqrF¢11	pBAD/Myc-His bearing truncated *nqrF* without nucleotides 1–72; Ap^r	21
pAL_sat7	pAL-TA bearing *K. pneumoniae nqrM* gene; Ap^r	This work
p15B_nqrmWT	Plasmid bearing the *V. harveyi nqrM*$_1$ gene; p15A *ori* Cm^r	This work
p15B_nqrmC33S	p15B_nqrmWT; C33S substitution in NqrM	This work
p15B_nqrmC45S	p15B_nqrmWT; C45S substitution in NqrM	This work
p15B_nqrmC47S	p15B_nqrmWT; C47S substitution in NqrM	This work
p15B_nqrmC51S	p15B_nqrmWT; C51S substitution in NqrM	This work

Fe ion coordinated by four cysteine residues (the Cys_4[Fe] center) between subunits NqrD and NqrE (13), and two covalently bound flavin mononucleotide (FMN) residues in subunits NqrB and NqrC (14, 15). The recently determined crystal structure of Na^+-NQR includes the whole set of cofactors (13). The flavins are attached posttranslationally via Thr residues under the action of flavin transferase (ApbE) encoded by an *nqr*-associated *apbE* gene (16). This reaction occurs on the periplasmic side of the bacterial membrane (17) and renders the enzyme active in quinone reduction (16).

However, our attempts to coexpress the *Vibrio harveyi nqr* operon and *apbE* gene in *Escherichia coli* failed to produce Na^+-NQR capable of Na^+-dependent quinone reduction. The logical corollary is that an additional *V. harveyi* protein (or proteins) is required for Na^+-NQR maturation. Here we identify such a protein (designated DUF539 in current databases) in *V. harveyi* and other *nqr*-containing bacteria and show that coexpression of its gene with *nqr* and *apbE* in *E. coli* does result in a fully functional Na^+-NQR. Based on our findings, we suggest renaming the protein and its gene to NqrM (for Na^+-NQR maturation) and *nqrM*, respectively.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The bacterial strains used in this study are listed in Table 1. *E. coli* and *Klebsiella pneumoniae* cells were routinely grown at 37°C in LB medium containing either 100 μg ampicillin, 10 μg tetracycline, 50 μg kanamycin, 100 μg erythromycin, and 20 μg chloramphenicol or 150 μg ampicillin, 3.3 μg tetracycline, 100 μg kanamycin, and 40 μg chloramphenicol, respectively, per 1 ml. All genetic manipulations were carried out using *E. coli* XL1-Blue and SM10λpir strains. *V. harveyi* cells were grown at 32°C in FM medium (18) containing 3.3 μg tetracycline, 100 μg kanamycin, 10 μg erythromycin, 100 μg rifampin, and 4 μg chloramphenicol per 1 ml.

Construction of *nqr*-containing plasmids. Different *nqr*-containing sequences were amplified from genomic DNA of *V. harveyi* R3, a rifampin-resistant strain selected from *V. harveyi* ATCC 33843 (B 392), by PCR using long-reading hot-start Encyclo polymerase (Evrogen). For all PCRs, the same forward primer 1F (see Table S1 in the supplemental material) was used, which included an in-frame stop codon and the translation reinitiation ATG sequence of the *nqrA* gene. The primer was designed to achieve production of authentic enzyme without the N-terminal leader sequence from the pBAD-TOPO TA vector (Invitrogen), in accordance with the manufacturer's manual.

The DNA fragments containing the *V. harveyi nqr* operon alone, together with the downstream *apbE* gene or with the downstream *apbE* and *nqrM*$_1$ genes were amplified with 1F/2R, 1F/3R, or 1F/4R primer pairs. The DNA fragments were cloned into a pBAD-TOPO TA vector, resulting in the plasmids pNQ, pNQ_AE, and pNQ_AE_NqrM, respectively (Fig. 1). The correct insertion and orientation of the amplified sequences in the obtained plasmids were checked by restriction analysis.

To produce an in-frame deletion of the *apbE* gene in the pNQ_AE_NqrM plasmid, an additional site for AfeI was introduced into the 3′ region of this gene by using a QuikChange II site-directed mutagenesis kit (Stratagene) and the Afe_dir/Afe_rev primer pair (see Table S1 in the supplemental material for the forward primer sequence). Internal deletion of the *apbE* gene was achieved using the original and added AfeI sites, resulting in plasmid pNQ_NqrM.

Construction of *nqrM*-deficient *K. pneumoniae* and *V. harveyi* strains. A DNA fragment of *K. pneumoniae* NTUH-K2044 containing the *nqrM* gene (kp1_1090, GenBank accession number BAH61889) was amplified by PCR with *Taq* polymerase and primers 7F/8R using the genomic DNA as a template. The amplified 641-bp fragment was cloned into the pAL-TA vector (Evrogen), resulting in plasmid pAL_sat7. A kanamycin

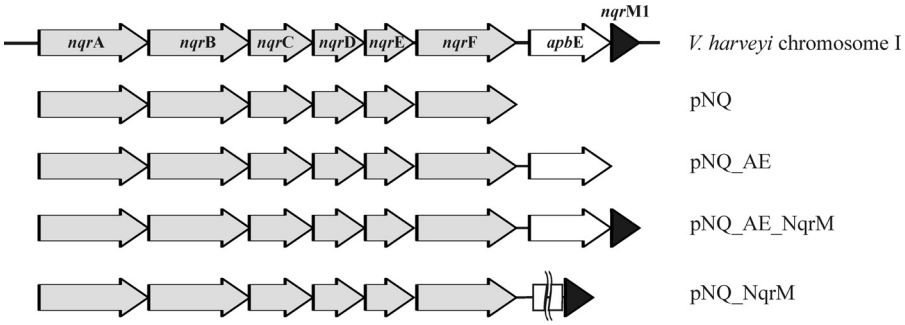

FIG 1 Arrangement of Na^+-NQR-associated genes in the *V. harveyi* ATCC 33843 chromosome (39) and the constructed vectors. The last construct contains a disrupted *apbE* gene. GenBank accession numbers: NqrA to -F: AIV06488 to AIV06483; ApbE, AIV06482; $NqrM_1$, AIV06481.

resistance cassette was inserted into the NruI site of the *nqrM* gene in pAL_sat7, and a plasmid (pALsatKm) bearing the *nqrM* gene together with the unidirectionally transcribing kanamycin resistance cassette was selected. The *nqrM*::Km^r fragment from pALsatKm was subcloned into the suicide vector pKNOCK-Tc, resulting in pKn282. This plasmid was transferred into the *K. pneumoniae* KNU210 (*nuoB*::Cm^r) strain via conjugation using *E. coli* SM10 λpir as the donor, and a Tc^s Km^r Cm^r phenotype clone (DUF539 [*nuoB*::Cm^r *nqrM*::Km^r]) bearing a double-crossover-introduced mutation was selected. Proper localization of the mutation in the *K. pneumoniae* chromosome was verified by PCR analysis. To complement *K. pneumoniae* DUF539 (*nqrM*::Km^r), the *nqrM*-carrying plasmid pAL_sat7 was transferred into the strain using electroporation.

V. harveyi ATCC 33843 contains two putative *nqrM* genes, one ($nqrM_1$) adjacent to the *nqr* operon on chromosome I and the other ($nqrM_2$) on chromosome II. To construct an $nqrM_1$::Cm^r *V. harveyi* strain, two DNA fragments containing upstream and downstream regions of the $nqrM_1$ gene (GenBank accession number AIV06481) were amplified using genomic DNA of *V. harveyi* R3 as the template and the primer pairs VH_DM_dir/VH_inR1 and VH_inD1/VH_DM_rev, respectively. The resulting 504- and 466-bp amplicons were combined by overlap extension PCR (19). The amplified 950-bp fragment was cloned into a pGEM-T vector (Promega), resulting in plasmid pG_DM3. The structure of the cloned fragment was confirmed by sequencing. A chloramphenicol resistance cassette was inserted into the Bpu14I site of the $nqrM_1$ gene in pG_DM3, and a plasmid (pG_DMCm) bearing $nqrM_1$ together with the unidirectionally transcribing chloramphenicol resistance cassette was selected. The $nqrM_1$::Cm^r fragment from pG_DMCm was subcloned into the suicide vector pKNOCK-Tc, resulting in pKn0512. This plasmid was transferred into the *V. harveyi* VHtag60 strain via conjugation using *E. coli* SM10 λpir as the donor, and a Tc^s Km^r Cm^r phenotype clone (VHDM15 [$nqrM_1$::Cm^r]) bearing a double-crossover-introduced mutation was selected. Proper localization of the mutation in the *V. harveyi* VHDM15 strain was verified by PCR analysis.

To produce an $nqrM_1$::Cm^r $nqrM_2$::Em^r double mutant *V. harveyi* strain, a DNA fragment containing the $nqrM_2$ gene (GenBank accession number AIV07656) was amplified by PCR with the primers VH_*nqrM2*_dir and VH_*nqrM2*_rev using the genomic DNA of *V. harveyi* R3 as a template. The amplified 3,417-bp fragment was cloned into the pGEM-T vector, resulting in the plasmid pG_LD2. An erythromycin resistance cassette was inserted directly into the $nqrM_2$ gene of the plasmid pG_LD2 utilizing the overlap extension PCR cloning method (20). The plasmid pG_LD2Erm was thereby produced using the primer pair up_OE_Erm and low_OE_Erm. The 2,995-bp NruI-EcoRI fragment bearing $nqrM_2$::Em^r from pG_LD2Erm was subcloned into the suicide vector pKNOCK-Tc, resulting in pKn210515. The structure of the subcloned fragment was confirmed by sequencing. The pKn210515 plasmid was transferred into the *V. harveyi* VHDM15 ($nqrM_1$::Cm^r) strain via conjugation using *E. coli* SM10 λpir as the donor, and the Tc^s Km^r Cm^r Em^r phenotype clone VHDDM109 ($nqrM_1$::Cm^r $nqrM_2$::Em^r) bearing a double-crossover-introduced mutation was selected. Proper localization of the mutation in the *V. harveyi* VHDDM109 strain was verified by PCR analysis, which showed the presence of Em^r cassette-disrupted $nqrM_2$ and the absence of the wild-type $nqrM_2$, despite the fact that the latter DNA element is significantly shorter and therefore should have been amplified with higher efficiency.

Cloning of the *V. harveyi* $nqrM_1$ gene in a p15A *ori* Cm^r plasmid. A DNA fragment containing $nqrM_1$ was amplified by PCR with the high-fidelity Tersus PCR kit (Evrogen) and primers 9F/10R using the genomic DNA of *V. harveyi* R3 as the template. The amplified 1,038-bp fragment was treated with DraI and HincII restriction endonucleases, and the 573-bp DraI-HincII fragment was ligated into plasmid p15BCM7 (16) digested with HincII. This resulted in construction of an $nqrM_1$-bearing p15A *ori*-dependent plasmid (p15B_nqrmWT). The fidelity of the construct was confirmed by DNA sequencing.

Site-directed mutagenesis of the *V. harveyi* $nqrM_1$ gene. The mutagenic primers to replace Cys33, Cys45, Cys47, and Cys51 in $nqrM_1$ with Ser are listed in Table S1 in the supplemental material. Mutagenesis was carried out using the QuikChange II kit (Stratagene) and plasmid p15B_nqrmWT as the template. The generated mutant plasmids (p15B_nqrmC33S, p15B_nqrmC45S, p15B_nqrmC47S, and p15B_nqrmC51S) were checked by DNA sequencing.

Preparation of membrane vesicles from *E. coli* and *K. pneumoniae* cells. The cells were harvested by centrifugation (10,000 × *g*, 10 min) and washed twice with medium 1 (100 mM KCl, 10 mM Tris-HCl, 5 mM $MgSO_4$, pH 8.0). The cell pellet was suspended in medium 2 (20 mM HEPES-Tris, 5 mM $MgSO_4$, 50 mM KCl, pH 8.0), and the suspension was passed through a French press (16,000 lb/in^2). Undamaged cells and cell debris were removed by centrifugation at 22,500 × *g* (10 min), and the supernatant was further centrifuged at 180,000 × *g* (60 min). The membrane pellet was then suspended in medium 2 (at 20 to 30 mg protein/ml) and used immediately for activity measurements. The protein concentration was determined by the bicinchoninic acid method using bovine serum albumin as the standard.

Isolation of the truncated NqrF subunit and complete Na^+-NQR complex. Plasmid pBAD_NqrF′11 encoding the truncated NqrF subunit (NqrF′), the soluble variant of *V. harveyi* NqrF containing its Fe-S and FAD-binding domains (21), was transferred into *E. coli* BL21 cells with or without plasmid p15B_nqrmWT. For *nqrF′* and $nqrM_1$ induction, the cells were grown at 32°C to mid-exponential phase (A_{600} = 0.3 to 0.4), after which the medium was supplemented with 0.2% (wt/vol) L-arabinose and growth was continued for 3 h. The cells were harvested by centrifugation (10,000 × *g*, 10 min), washed twice with medium containing 300 mM NaCl, 5 mM $MgSO_4$, and 10 mM Tris-HCl (pH 8.0), and suspended in medium containing 300 mM NaCl, 5 mM $MgSO_4$, 1 mM phenylmethylsulfonyl fluoride, 20 mM Tris-HCl, and 5 mM imidazole-HCl (pH 8.0). The suspension was passed twice through a French press at 16,000 lb/in^2, cell debris and membrane vesicles were removed by centrifugation at 180,000 × *g* (60 min), and 6×His-tagged NqrF′ was purified

FIG 2 Sequence alignments of typical NqrM proteins of proteobacteria using the MUSCLE program (40). The sequences were detected as hits in a BLAST search of complete prokaryotic genomes using the *V. harveyi* $NqrM_1$ protein (GenBank accession number AIV06481) as the query. The GenBank accession numbers are indicated in parentheses. Predicted transmembrane α-helices are shown as gray boxes, consensus residues are indicated below the set of sequences, and four invariant Cys residues are marked by black boxes.

from the supernatant using affinity chromatography. This was accomplished by loading the supernatant onto an Ni-nitrilotriacetic acid (NTA) column equilibrated with 5 mM imidazole-HCl (pH 8.0), washing the column with 10 mM imidazole-HCl, and eluting the 6×His-tagged protein with 100 mM imidazole-HCl. All the buffers used in the affinity chromatography additionally contained 300 mM NaCl and 10 mM Tris-HCl (pH 8.0). 6×His-tagged Na^+-NQR was isolated from *V. harveyi* strain VHtag60 or VHDDM109 and purified using affinity chromatography as described previously (22). The proteins thus obtained were concentrated and kept frozen in liquid N_2 until use.

Activity assays. NADH and dNADH oxidation by membrane vesicles isolated from *E. coli* or *K. pneumoniae* strains was measured at 30°C using a Hitachi 557 spectrophotometer at 340 nm. The quinone reductase activity of Na^+-NQR was measured by following the sodium-stimulated component of oxidation of the potassium salt of dNADH. The reaction medium contained 20 mM HEPES-Tris, 5 mM $MgSO_4$, and 50 mM KCl (pH 8.0). For measurement of dNADH:menadione oxidoreductase activity [(d)NADH dehydrogenase activity of Na^+-NQR], the reaction medium was supplemented with 50 μM menadione. An extinction coefficient, ε_{340}, of 6.22 mM^{-1} cm^{-1} was used for NADH and dNADH quantitation. The potassium salt of dNADH was prepared as described previously (17). NADH dehydrogenase and quinone reductase activities of the purified Na^+-NQR preparations were measured as described previously (14). All reported activity values are the means of three measurements ± standard deviation (SD).

MS. 6×His-tagged Na^+-NQR (0.1 mg) was precipitated with 7.5% (vol/vol) trifluoroacetic acid, sedimented by centrifugation, washed with acetonitrile, and dissolved in 100 μl H_2O. A 1-μl aliquot of the solution was mixed with 0.5 μl of a saturated solution of 2,5-dihydroxybenzoic acid in 20% acetonitrile with 0.5% trifluoroacetic acid on a steel target plate. Mass spectra (MS) were recorded on an Ultraflex Extreme matrix-assisted laser desorption ionization–tandem time of flight (MALDI-TOF/TOF) mass spectrometer (Bruker Daltonics, Germany) equipped with an Nd laser (354 nm). The $[MH]^+$ molecular ions were detected in a linear mode in the *m/z* ranges of 2,000 to 20,000 and 15,000 to 100,000; the values of peak masses were accurate to 20 Da.

RESULTS

Genes associated with the *nqr* operon in different bacteria. Genes of functionally related polypeptides are often clustered in bacterial genomes (23). Thus, the six genes encoding the Na^+-NQR subunits are commonly organized into a single *nqrABCDEF* operon (9, 10) joined by an *apbE* gene, whose product carries out flavinylation of the NqrB and NqrC subunits (16). Our analysis of sequenced bacterial genomes has additionally pinpointed an adjacent putative gene, *nqrM* (formerly designated *duf539*), whose product, NqrM, with an unknown function belongs to the InterPro domain family IPR007495 (Pfam PF04400, COG COG2991). A BLAST search among all sequenced bacterial genomes (RefSeq database, September 2015) using NqrM from *V. harveyi* (accession number AIV06481) as a query has revealed *nqrM* only in 977 *Proteobacteria* and 13 *Planctomycetes* having the Na^+-NQR genes (only one genome was analyzed for each unique retrieved NqrM sequence). Most *Vibrionaceae* and *Psychromonadaceae* as well as *Plesiomonas shigelloides* possess two slightly different putative *nqrM* genes, one ($nqrM_1$) adjacent to the *nqr* operon and the other ($nqrM_2$) usually adjacent to the gene whose product is annotated as dinitrogenase iron-molybdenum cofactor domain protein. Bacteria of other taxa, including *Shewanellaceae* with duplicated *nqr* operons, have only one copy of *nqrM*. Most commonly, *nqrM* follows *apbE* and the *nqr* operon (Fig. 1), but it may follow the *nqr* operon in cases where *apbE* is distantly located on the chromosome (e.g., in *K. pneumoniae*). In only rare cases is the *apbE-nqrM* pair at a distance from the *nqr* operon (e.g., in *Haemophilus parasuis* and some *Neisseria* species) or are all three partners at a distance from one another (e.g., in *Leucothrix mucor* and *Thauera* and *Moraxella* species). Interestingly, the *nqr* operon is followed by the *apbE* gene fused with the *nqrM* gene in *Nitrosomonas europaea* ATCC 19718 and several *Rhodopirellula* species.

The coexistence and colocalization of *nqrM*, the *nqr* operon, and *apbE* suggested a functional relationship between NqrM and the Na^+-NQR/ApbE pair. Of note, *nqrM* is not specifically coupled with the *rnf* operon, which encodes Na^+-dependent NADH:ferredoxin oxidoreductase, an enzyme, similar to Na^+-NQR. Thus, *nqrM* is found in *Enterobacteriaceae* containing both *nqr* and *rnf* operons (e.g., in *Yersinia pestis* and *K. pneumoniae*) but is never observed in those containing only the *rnf* operon (e.g., in *E. coli* and *Salmonella enterica*).

NqrM is a small, moderately conserved protein formed by 60 to 80 amino acid residues (Fig. 2). Its N-terminal part invariably contains a hydrophobic 20-residue sequence that is predicted by the TMHMM (24) and TMpred (25) programs to form a single putative transmembrane α-helix. The C-terminal part is formed predominantly by hydrophilic amino acids. Most interestingly, all NqrM proteins contain four Cys residues, except for *Endozoicomonas* NqrM, which has only three Cys residues.

In *Gammaproteobacteria*, the Cys residues are generally arranged in two ways in the sequence. The motif $CX_{11}CXCXXXC$, where X is any residue, is found in *Aeromonadales*, *Vibrionales*, *Enterobacteriaceae*, *Cardiobacteriales*, *Chromatiales*, *Pasteurellales*, and most *Alteromonadales* (Fig. 2). However, this motif is replaced by $CX_{11}CXXCX_6C$ in *Pseudomonadales*, *Oceanospirillales*, *Cellvibrionales*, and part of *Alteromonadales*. Despite a large variability of Cys arrangement in other *Proteobacteria* (there are in total 25 such arrangements), all NqrM proteins share an N-terminal hydrophobic α-helix and four Cys residues in the hydrophilic part and demonstrate sequence similarity in the region between the α-helix and the first Cys residue, including its immediate environment (Fig. 2).

Although *nqrM* is found only in genomes having the Na^+-

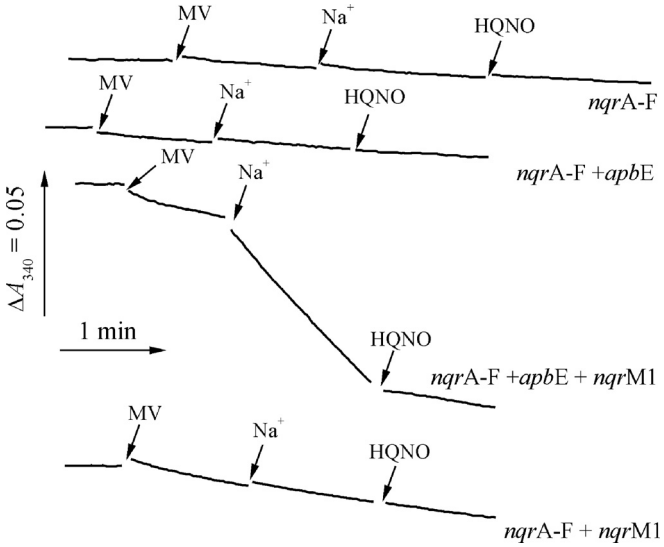

FIG 3 Typical traces of dNADH oxidation obtained in the assay of quinone reductase activity of Na^+-NQR. Membrane vesicles were prepared from *E. coli* cells expressing different combinations of *V. harveyi* genes indicated by the curve labels. Arrows indicate additions of 80 to 100 μg membrane vesicles (MV), 30 mM NaCl (Na^+), or 3 μM HQNO.

NQR genes, the reverse is not true. In *Bacteroidetes*, the *nqr* operon is accompanied instead by an *nqrM*-like gene that may have a reduced number of conserved Cys residues (see Fig. S1 in the supplemental material). Interestingly, NqrM or an NqrM-like factor is missing in some Na^+-NQR-containing *Deltaproteobacteria*, as well as in chlamydiae, *Deferribacter*, *Denitrovibrio*, and some other bacteria. This finding may mean that these bacteria use a different factor in Na^+-NQR maturation.

Heterologous expression of *V. harveyi* Na^+-NQR-associated genes in *E. coli*. In order to identify the minimal set of additional *V. harveyi* proteins required to produce fully functional *V. harveyi* Na^+-NQR in *E. coli* cells, we prepared four types of vector constructs, all containing the *nqrA* to *-F* genes encoding six Na^+-NQR subunits. One construct (pNQ [Fig. 1]) contained only the *nqr* operon, two other constructs additionally contained the *apbE* gene (pNQ_AE) or both the *apbE* and $nqrM_1$ genes (pNQ_AE_NqrM), and the last construct (pNQ_NqrM) combined the genes for Na^+-NQR, ApbE, and $nqrM_1$, but the *apbE* gene was not functional. We amplified the corresponding *V. harveyi* genomic DNA fragments by PCR and cloned them into a pBAD expression vector. Disruption of the *apbE* gene in the pNQ_NqrM plasmid was carried out by in-frame deletion of the gene in pNQ_AE_NqrM. All produced constructs were expressed in *E. coli* ANN091 (*nuoI*::Km^r) cells. This strain is deficient in H^+-translocating NADH:quinone oxidoreductase (NDH-1) and cannot oxidize dNADH (26, 27). Hence, the quinone reductase and NADH dehydrogenase activities of *V. harveyi* Na^+-NQR could be characterized in *E. coli* membrane vesicles by measuring dNADH oxidase and dNADH:menadione oxidoreductase activities, respectively.

The activities of the recombinant strains thus produced are shown in Fig. 3 and Table 2. All strains demonstrated high dNADH:menadione oxidoreductase activity, which was absent in the host cells bearing an empty pBAD plasmid and is thus attributable to *V. harveyi* Na^+-NQR. However, Na^+-stimulated, HQNO-inhibited dNADH oxidase activity was observed only in *E. coli* cells expressing the whole set of functional genes, including *apbE* and $nqrM_1$. Furthermore, expression of any of the pNQ, pNQ_AE, and pNQ_AE_NqrM constructs in *V. cholerae* O395N1 Δ*nqrABCDEF* cells that lacked the whole *nqr* operon (28) resulted in complete recovery of the Na^+-NQR activity (data not shown), confirming the intactness of the *nqr* genes in the constructed plasmids. These findings indicated that production of *V. harveyi* Na^+-NQR capable of quinone reduction in *E. coli* critically depends on the presence of the *V. harveyi* ApbE and $NqrM_1$ proteins.

TABLE 2 Enzymatic activities of *E. coli* ANN091 (*nuoI*::Km^r) cells transformed with plasmids containing different *V. harveyi* genes or with an empty pBAD plasmid

Plasmid	Activity in membrane vesicles, nmol · min^{-1} · mg^{-1} (mean ± SD)		
	Na^+-stimulated dNADH oxidase	dNADH:menadione oxidoreductase	NADH oxidase
pNQ	<1	240 ± 70	830 ± 120
pNQ_AE	3 ± 1	260 ± 40	770 ± 90
pNQ_AE_NqrM	65 ± 8	310 ± 60	850 ± 200
pNQ_NqrM	<1	220 ± 60	790 ± 50
pBAD	<1	5 ± 2	750 ± 80

Mutational analysis of *nqrM* in *K. pneumoniae* and *V. harveyi* cells. The above analysis involved gene transfer between different bacteria. Here, we determined how *nqrM* inactivation affects Na^+-NQR activity in the same cell. *K. pneumoniae* was originally selected for such an analysis, as its genome contains only one *nqrM* gene (kp1_1090, GenBank accession number BAH61889). Furthermore, the *K. pneumoniae* KNU210 strain is deficient in the *nuoB* gene, which encodes a subunit of the H^+-translocating NADH:quinone oxidoreductase (NDH-1). Accordingly, Na^+-NQR is the only dNADH-oxidizing complex in this strain (29), which again facilitated the activity assay. As Table 3 makes clear, mutation in *nqrM* nearly completely abolished the Na^+-stimulated dNADH oxidation by Na^+-NQR in *K. pneumoniae* without any significant effect on its dNADH:menadione oxidoreductase activity and the activities of other respiratory chain enzymes (as characterized by the differences between the NADH and dNADH oxidase activities). A plasmid containing the intact *nqrM* gene (kp1_1090) nearly completely restored the Na^+-stimulated (d)NADH oxidase activity of Na^+-NQR (Table 3), ruling out a possible polar effect of the mutation.

As already mentioned, the *V. harveyi* genome contains genes for two NqrM proteins ($nqrM_1$ and $nqrM_2$; GenBank accession numbers AIV06482 and AIV07656, respectively), complicating mutagenesis analysis. Therefore, we inactivated first only the *nqr*-adjacent $nqrM_1$ gene and then both *nqrM* genes. Inactivation of

TABLE 3 Enzymatic activities of mutated *K. pneumoniae* KNU210 cells

Mutation	Activity in membrane vesicles, nmol · min^{-1} · mg^{-1} (mean ± SD)		
	Na^+-stimulated dNADH oxidase	dNADH:menadione oxidoreductase	NADH oxidase
nuoB::Cm^r	97 ± 8	230 ± 25	280 ± 30
nuoB::Cm^r *nqrM*::Km^r	2.4 ± 1.6	100 ± 10	190 ± 4
nuoB::Cm^r *nqrM*::Km^r/ pAL_sat7	72 ± 6	180 ± 20	250 ± 25

TABLE 4 Enzymatic activities of *V. harveyi* VHtag60 cells with the indicated mutations

Mutation	Activity in membrane vesicles, nmol · min^{-1} · mg^{-1} (mean ± SD)		
	Na^+-stimulated dNADH oxidase	dNADH:menadione oxidoreductase	NADH oxidase
None	720 ± 80	1,300 ± 140	1,600 ± 250
$nqrM_1$::Cm^r	210 ± 30	1,100 ± 140	1,400 ± 210
$nqrM_1$::Cm^r $nqrM_2$::Em^r	<5	900 ± 150	1,100 ± 180

$nqrM_1$ resulted in a 3.5-fold decrease in the Na^+-stimulated dNADH oxidase activity of *V. harveyi* cells without any significant effect on their dNADH:menadione oxidoreductase activity and the activities of other respiratory chain enzymes (Table 4). Inactivation of both *nqrM* genes completely abolished the Na^+-stimulated dNADH oxidase activity. These findings indicated that both NqrM proteins may be involved in production of the fully functional Na^+-NQR complex in *V. harveyi*. Importantly, inactivation of the *nqrM* genes in *K. pneumoniae* and *V. harveyi* did not result in a decrease in dNADH:menadione oxidoreductase activity associated with the NqrF subunit (Tables 3 and 4). As all Na^+-NQR genes are combined in one operon, these results ruled out the possibility that NqrM is involved in transcription regulation of the *nqr* genes.

Isolation of Na^+-NQR from the authentic and *nqrM*-deficient *V. harveyi* strains. The Na^+-NQR complex containing a 6×His-tagged NqrF subunit was isolated by metal chelate chromatography from the parental *V. harveyi* VHtag60 strain (22) and its VHDDM109 variant strain containing lesions in both *nqrM* genes. Purification of Na^+-NQR from the VHtag60 strain resulted in an enzyme complex that contained the full set of subunits (Fig. 4A) and was fully active in both dNADH oxidation and Na^+-dependent quinone reduction. In contrast, the protein isolated by the identical procedure from the VHDDM109 strain lacked the NqrB, the NqrD, and possibly the NqrA and NqrE subunits (Fig. 4B). The yield of the incomplete Na^+-NQR complex in terms of the protein content was 13-fold lower, despite the larger content of contaminating proteins in it (Fig. 4). The incomplete Na^+-NQR complex exhibited high NADH dehydrogenase activity (12 μmol · min^{-1} · mg^{-1}), attributable to the NqrF subunit (4), but no Na^+-stimulated quinone reductase activity. These data indicated that NqrM inactivation somehow prevented assembly of a complete Na^+-NQR complex.

Notably, the MALDI spectrum of isolated fully functional Na^+-NQR contained no peak corresponding to NqrM (8,291 Da) (Fig. 4A). To corroborate this result, the same preparation of *V. harveyi* Na^+-NQR was separated by Tricine-SDS-PAGE (30), the gels were stained with Coomassie blue, and all detected protein bands with masses less than 12 kDa were analyzed by MALDI MS. Again, neither band showed identity with NqrM. Thus, NqrM does not represent a previously unrecognized Na^+-NQR subunit, in agreement with the three-dimensional structure of the enzyme (13).

NqrM is not required for [2Fe-2S] cluster formation in subunit NqrF. The cytoplasmically oriented subunit NqrF of Na^+-NQR contains two catalytically important cofactors: a [2Fe-2S] cluster and a noncovalently bound FAD molecule. To make NqrF water soluble, its single transmembrane α-helix found in the N-terminal portion can be eliminated by genetic manipulations (4, 21). For a rigorous test for the role of NqrM in [2Fe-2S] cluster formation, this truncated NqrF (NqrF′) was produced in *E. coli* cells transformed with the *nqr*F′-bearing plasmid alone or in combination with the p15B_nqrmWT plasmid bearing $nqrM_1$. Purification of NqrF′ from these cells yielded brownish-yellow proteins that were detected as 45-kDa bands (calculated mass of the 6×His-tagged NqrF′ polypeptide, 45.8 kDa) in SDS-polyacrylamide gels (data not shown).

The electron paramagnetic resonance (EPR) spectra of both NqrF′ preparations revealed a nearly axial signal with characteristic g values ($g_{||}$ = 2.02 and $g_{\perp}$ = 1.94) (Fig. 5) that were identical to the signal of the Na^+-NQR [2Fe-2S] cluster (31). Spin concentrations normalized to the same amount of protein were similar for the two NqrF′ preparations, indicating that the [2Fe-2S] clus-

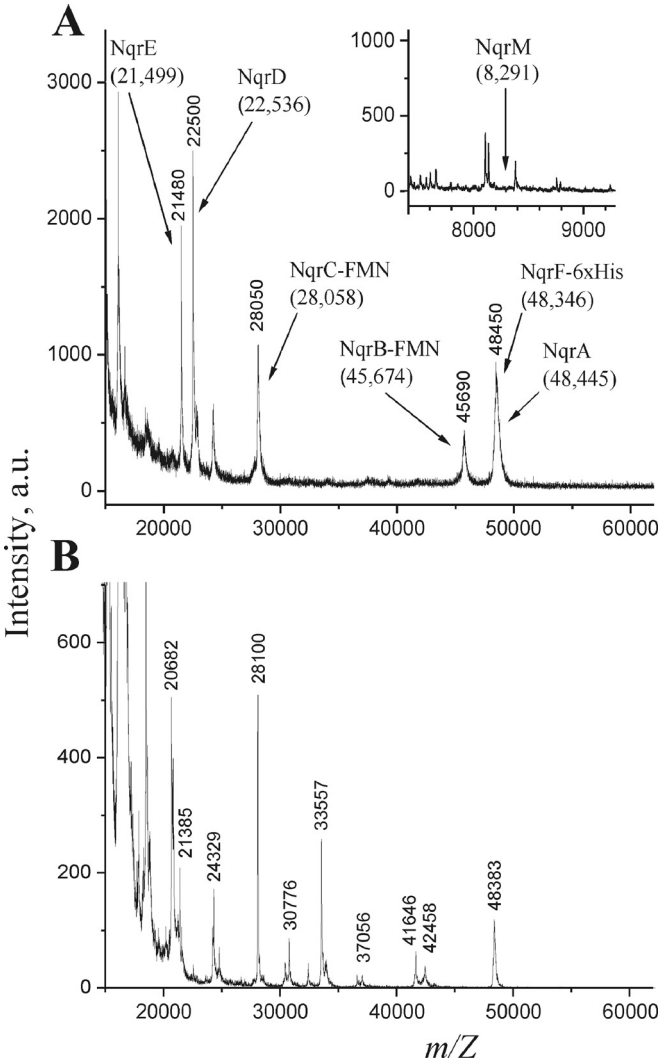

FIG 4 MALDI mass spectral analysis of Na^+-NQR isolated from the authentic *V. harveyi* VHtag60 strain (A) and its $nqrM_1$-deficient variant VHDDM109 (B). The inset shows data collected in a different *m/z* range. Arrow labels indicate identified subunits with their added moieties and predicted averaged molecular masses (in parentheses). Predicted masses of the NqrC and NqrB subunits are for polypeptides without the N-terminal methionines (8). Peak sizes are not directly comparable between panels A and B because of the inherent features of mass spectral analysis.

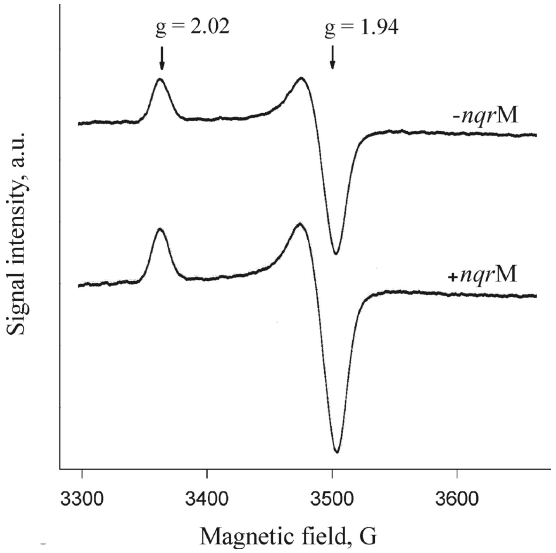

FIG 5 EPR spectra of isolated *V. harveyi* Na^+-NQR subunit NqrF′ expressed in *E. coli* with or without the *V. harveyi* $nqrM_1$ gene. The spectra of dithionite-reduced NqrF′ were recorded on a Bruker ESC-106 X-band EPR spectrometer at a temperature of 77 K, a microwave frequency of 9.51 GHz, 20-mW microwave power, and 0.5-mT modulation amplitude. The NqrF′ concentrations were 50 and 65 μM for the upper and lower traces, respectively.

ter can be formed independently of the NqrM protein. Notably, FAD was similarly found in NqrF′ produced in the absence or presence of NqrM (data not shown).

Directed mutagenesis of Cys residues in *V. harveyi* NqrM. To investigate the roles of NqrM Cys residues in Na^+-NQR production, the *V. harveyi* $nqrM_1$ gene was cloned into an expression vector to yield plasmid p15B_nqrmWT. Cys/Ser substitutions in NqrM were achieved by making appropriate replacements in this plasmid. Coexpression of the p15B_nqrmWT plasmid with the pNQ_AE plasmid bearing the *V. harveyi nqr* operon and *apbE* gene in *E. coli* ANN091 cells induced appreciable Na^+-stimulated, HQNO-sensitive dNADH oxidase activity, i.e., production of the functional Na^+-NQR complex (Table 5). As the transcription of $nqrM_1$ and *nqr* is under the control of the *araBAD* promoter in the above constructs, expressions was induced at different L-arabinose concentrations in order to vary the NqrM and Na^+-NQR levels in the cells. The activity of Na^+-NQR coproduced with wild-type NqrM was an order of magnitude higher in cells grown in the presence of L-arabinose than in cells grown in its absence. Moreover, all four Cys/Ser substitutions in NqrM resulted in either zero or very low quinone reductase activity by Na^+-NQR in cells grown without L-arabinose.

For a more sensitive test for the importance of particular Cys residues, similar measurements were performed with cells producing higher levels of NqrM (i.e., grown with 0.2% L-arabinose), which was expected to partially compensate for the effects of the Cys/Ser substitutions on the activity. As shown in Table 5, the C33S variant again demonstrated zero activity, whereas the other variants showed significant, though reduced, activities. These data support the role of NqrM in Na^+-NQR production and suggest that Cys33 is absolutely required for NqrM function. The three other Cys residues appear to be less important, as the variant proteins were partially active.

DISCUSSION

More than 20% of predicted protein sequences are currently annotated as "domains with unknown function" (DUFs) in sequence databases. Accordingly, identification of their physiological functions represents an important goal of modern molecular biology. In this work, we demonstrated that one such unidentified protein, DUF539, which is here renamed NqrM, is a maturation factor for Na^+-NQR in *V. harveyi*. Only two Na^+-NQR-specific factors, NqrM and the flavin transferase encoded by the *apbE* gene, are required to produce fully functional *V. harveyi* Na^+-NQR in *E. coli* cells that lack their own *nqr* operon and *nqrM*. Though *E. coli* contains its own *apbE* gene, its product is not functional with *V. harveyi* Na^+-NQR (16).

The function of NqrM in Na^+-NQR maturation apparently depends on the four conserved Cys residues. The observation that replacements of three of these residues by Ser do not cancel out NqrM function rules out the possibility that they maintain protein structure by forming intramolecular disulfide bonds. That only a Cys/His replacement is tolerated for only one of these residues (see the supplemental material) suggests that they serve as ligands for a metal ion or metal cluster used to build up the Na^+-NQR molecule. Based on the structures available in the Protein Data Bank (PDB), the four-Cys scaffold can accommodate either an Fe-S cluster or a single Fe ion. Well-known examples of corresponding small proteins are adrenodoxin (PBD 2MJE) (32), mediating electron transport from the NADPH-dependent adrenodoxin reductase to mitochondrial cytochromes P450, and rubredoxin (PDB code 4RXN) (33). A membrane form of the latter protein, rubredoxin A, is required for maturation of

TABLE 5 Effects of Cys substitutions in *V. harveyi* $NqrM_1$ on the enzymatic activity of *V. harveyi* Na^+-NQR coproduced in *E. coli*

	Activity in membrane vesicles, nmol · min^{-1} · mg^{-1} (mean ± SD)[a]					
	Na^+-stimulated dNADH oxidase		dNADH:menadione oxidoreductase		NADH oxidase	
Substitution	Without L-arabinose	With L-arabinose	Without L-arabinose	With L-arabinose	Without L-arabinose	With L-arabinose
None	4.7 ± 1.1	53 ± 10	18 ± 4	250 ± 80	580 ± 50	720 ± 90
C33S	<0.1	<1	15 ± 4	190 ± 70	620 ± 70	690 ± 80
C45S	0.4 ± 0.1	27 ± 3	24 ± 6	280 ± 100	510 ± 50	670 ± 20
C47S	<0.1	28 ± 2	15 ± 1	220 ± 80	550 ± 90	730 ± 130
C51S	<0.1	19 ± 2	25 ± 8	300 ± 60	610 ± 80	700 ± 100
No-*nqrM* control[b]	<0.1	<1	16 ± 6	250 ± 30	710 ± 80	700 ± 90

[a] Membranes were obtained from cells grown without L-arabinose or with 0.2 % L-arabinose in the culture medium, i.e., at low and high levels of *V. harveyi* $nqrM_1$ and *nqr* expression, respectively.
[b] Cells were transformed with the pNQ_AE plasmid (bearing the *nqr* and *apbE* genes) and an empty pBAD plasmid containing p15A *ori*.

photosystem I and resembles NqrM in having a transmembrane α-helix (34). Of note, NqrM is not required for the ApbE-catalyzed flavinylation of Na^+-NQR, as this reaction proceeds readily in *E. coli* in the absence of NqrM (16).

Experiments with the water-soluble form of the *V. harveyi* NqrF subunit indicated, at variance with the data of Tao et al. (35), that this subunit can be separately expressed in *E. coli* and that its [2Fe-2S] cluster can form in *E. coli* in the absence of NqrM. Indeed, *E. coli* and other bacterial cells contain the so-called iron-sulfur cluster (ISC) and sulfur mobilization (SUF) systems, which permit the maturation of various Fe/S proteins (36). Each system contains a cysteine desulfurase to produce sulfur from L-cysteine, a scaffold protein to form a cluster, and a carrier protein to deliver the cluster to the target. Based on these considerations, we suggest that NqrM is involved, possibly in combination with the ISC/SUF system, in Fe delivery to subunits NqrD and NqrE to form the Cys_4[Fe] center mediating electron transfer between the [2Fe-2S] cluster and an FMN residue in Na^+-NQR. In the framework of this hypothesis, the role of Cys_4[Fe] formation in Na^+-NQR assembly can be rationalized in terms of its importance for establishing a strong interaction between subunits NqrD and NqrE, each of which provides a pair of Cys residues to this center. Indirect support for this idea comes from the mutagenesis data showing that the Cys residues participating in the Cys_4[Fe] center are important for Na^+-NQR assembly (37).

The requirement for a specific protein to form the intersubunit Cys_4[Fe] center may result from the uniqueness of this structure. Recruitment of auxiliary proteins is not uncommon in the biogenesis of unique Fe/S proteins. An example of such an auxiliary component is the rubredoxin-related HoxR protein, which prevents oxidative damage to metallocenters during their biogenesis in the [NiFe] hydrogenase of *Ralstonia eutropha* H16 (38). In this context, NqrM may represent an additional, Na^+-NQR-specific protein that directly functions in Na^+-NQR maturation in the respective bacteria. However, a possibility that NqrM is a transcriptional regulator that guides expression of an Na^+-NQR-specific factor which, in turn, directly participates in Na^+-NQR maturation cannot be excluded at present. In any case, the uniqueness of NqrM suggests it as a possible drug target to combat bacterial pathogens (such as *Enterobacter aerogenes*, *Vibrio cholerae*, *Vibrio parahaemolyticus*, *Haemophilus influenzae*, or *Yersinia pestis*) that encode NqrM and apparently depend on it for their Na^+-NQR activity.

In summary, we identified a second specific factor, NqrM, that is involved, in addition to common protein synthesis machinery, in the maturation of Na^+-translocating NADH:quinone oxidoreductase in proteobacteria. Testing the hypothesis that NqrM delivers Fe to Na^+-NQR may provide further insight into the mechanism of its biogenesis.

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