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A *nhaD* Na^+/H^+ *antiporter* and a *pcd* homologues are among the *Rhodothermus marinus* complex I genes

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Abstract

The NADH:menaquinone oxidoreductase (Nqo) is one of the enzymes present in the respiratory chain of the thermohalophilic bacterium *Rhodothermus marinus*. The genes coding for the *R. marinus* Nqo subunits were isolated and sequenced, clustering in two operons [*nqo*$_1$ to *nqo*$_7$ (nqo$_A$) and *nqo*$_{10}$ to *nqo*$_{14}$ (nqo$_B$)] and two independent genes (*nqo*$_8$ and *nqo*$_9$). Unexpectedly, two genes encoding homologues of a NhaD Na^+/H^+ antiporter (NhaD) and of a pterin-4α-carbinolamine dehydratase (PCD) were identified within nqo$_B$, flanked by *nqo*$_{13}$ and *nqo*$_{14}$. Eight conserved motives to harbour iron–sulphur centres are identified in the deduced primary structures, as well as two consensus sequences to bind nucleotides, in this case NADH and FMN. Moreover, the open-reading-frames of the putative NhaD and PCD were shown to be co-transcribed with the other complex I genes encoded by nqo$_B$. The possible role of these two genes in *R. marinus* complex I is discussed.

Keywords: Complex I; NADH:quinone oxidoreductase; *Rhodothermus marinus*; NhaD Na^+/H^+ antiporter

1. Introduction

Complex I (NADH:quinone oxidoreductase, Nqo, NDH-1) catalyses the rotenone and piericidin A sensitive transfer of electrons from NADH to quinones, coupled to proton (or sodium) translocation across the membrane [1,2]. The bacterial complex I is usually composed of 14 subunits (Nqo$_1$ to Nqo$_{14}$/NuoA to NuoN), although in some bacteria fusions of Nqo subunits are observed (e.g., *Escherichia coli* [3] and *Aquifex aeolicus* [4]). It is generally accepted that the enzyme contains one non-covalently bound FMN. However, Albracht and co-workers proposed the existence of a second binding site for FMN in the bovine PSST subunit (Nqo$_6$ homologue) [5]. Whether this hypothesis can be extended to the complex I of other organisms is still a matter of debate. Nevertheless, to date, only two nucleotide-binding motives were pointed out in complex I subunits, both located within subunit Nqo$_1$ [6]. NDH-1 subunits generally contain eight conserved binding-sites for iron–sulphur clusters, for which EPR spectroscopy assigns six $[4Fe–4S]^{2+/1+}$ and two $[2Fe–2S]^{2+/1+}$ centres [7]. Nakamaru-Ogiso and co-workers characterised a ninth iron–sulphur centre in the Nqo$_3$ subunit of *Thermus thermophilus* complex I, a tetranuclear cluster [8], which is also predicted in the *E. coli* homologue [9], named N7. A tenth putative iron–sulphur centre binding-site is present in *Helicobacter pylori* [10]. However, these motives are not conserved in most of the available homologue sequences.

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The membrane arm subunits Nqo_{11-14} of complex I are homologous to subunits of the oligomeric Mrp (multiple resistance to pH) Na^+/H^+ antiporters: Nqo_{11} is homologous to MrpC, Nqo_{12} is homologous to MrpA, and Nqo_{13} and Nqo_{14} are homologous to MrpD [11]. In fact, it was demonstrated that the C-terminally truncated Nqo_{12} subunit of the *E. coli* complex I is able to carry out sodium transport [12].

The genomic organisation of Nqo is not strictly conserved among bacteria. For instance, it can be encoded by a single cluster, as in *T. thermophilus* [13] and *E. coli* [3], or by gene clusters spread in the genome, as observed in *A. aeolicus* [4].

Until now, there is no X-ray structure available for Nqo; nevertheless, electron microscopy data for the *E. coli*, *Neurospora crassa*, and *A. aeolicus* complexes [14,15] showed that complex I has an L-shape structure. It comprises two major domains, one hydrophobic arm imbedded in the cytoplasmic or the inner mitochondrial membrane, and a peripheral arm which protrudes into the cytoplasm, or the mitochondrial matrix, and contains the iron–sulphur clusters and the flavin of the enzyme [16].

Rhodothermus marinus, a bacterium from the *Flexibacter*, *Bacteroids* and *Cytophaga* group [17], is a microaerophilic organism, growing optimally at 65 °C and 2% NaCl. The energy conserving aerobic electron transport chain of *R. marinus* contains menaquinone-7, a cytochrome *c* and a HiPIP (High Potential Iron–sulphur Protein) [18], as electron carriers, and the enzymatic complexes Nqo [19], succinate:menaquinone oxidoreductase[20], cytochrome *bc* that oxidises menaquinone and reduces HiPIP [21], and can therefore replace the bc_1 complex, caa_3 HiPIP:oxygen oxidoreductase [22,23], and cbb_3 and ba_3 oxygen reductases [24]. The Nqo of *R. marinus* respiratory chain was isolated, and it was reported that the electron transfer from NADH to quinone analogues is coupled to the formation of a membrane potential. The enzyme has a non-covalently bound FMN, and several iron–sulphur centres were revealed by EPR spectroscopy [19].

The *R. marinus* complex I is one of the few examples of bacterial complexes I so far purified. Therefore it is essential to determine its primary structure. In the present work, the isolation and sequencing of the genes encoding *R. marinus* complex I is described. The unexpected presence of genes encoding a putative pterin-4α-carbinolamine dehydratase (PCD) and NhaD Na^+/H^+ antiporter (NhaD) homologues in the nqo operons is discussed.

2. Experimental procedures

2.1. Isolation and sequencing of nqo genes

DNA manipulation procedures were carried out according to [25]. The experimental strategy for the characterisation of the first nqo gene cluster, nqo_A, was as follows: degenerated oligonucleotides were designed based on the N-terminal sequence of subunit Nqo_1 (nqo_1f) from the purified *R. marinus* PRQ-62B complex I, and from an internal region of the sequence of the same subunit (nqo_1r), which is highly conserved among different bacteria (Table 1). A 562-bp PCR product was amplified from the *R. marinus* genomic DNA using the oligonucleotides Nqo_1f and Nqo_1r and *Taq* polymerase. The amplified product was cloned into pGEM-T Easy Vector (Promega) and its sequencing confirmed that the gene fragment deduced amino acid sequence corresponds to the internal region of the Nqo_1 subunit of complex I. The nqo_1 DNA fragment was then excised with *Eco*RI restriction enzyme, labelled with the DIG random-primer labelling system (Roche Molecular Biochemicals), and used to probe a *R. marinus* DNA library [23]. Upon isolation, a phage containing the complete nqo_1 gene was sequenced using a primer walking strategy (STABvida).

To obtain the sequence of nqo_B, a second gene cluster, degenerated oligonucleotides designed on the basis of two highly conserved regions of the Nqo_{12} subunit of complex I (nqo_{12}f and nqo_{12}r; Table 1), and used together with *R. marinus* genomic DNA in a PCR reaction, allowed the amplification of a 274-bp PCR product. The product was cloned in pZero (Invitrogen) and, after labelling, this fragment was used to search the gene encoding Nqo_{12} in the *R. marinus* DNA library.

Based on the nqo_8 and nqo_9 DNA sequences previously identified in *R. marinus* ITI-378 (G.O. Hreggvidsson and J.K. Kristjansson, unpublished results), primers nqo_8f and nqo_8r, and nqo_9f and nqo_9r (Table 1) were designed and used to amplify the nqo_8 and nqo_9 genes from *R. marinus* PRQ-62B genomic DNA.

2.2. DNA and protein sequence analyses

DNA sequence analyses were carried out using the Genetics Computer Group package (Wisconsin) provided by

Table 1
List of used oligonucleotides

Name	DNA sequence	Product size (bp)
nqo1f	5′-CSACSAAYGGNGCSCARWSSAARGC-3′	562
nqo1r	5′-SGTYTCYTCNCCRCARATRTASGC-3′	
nqo12f	5′-ATYCAYGCSGCSACSATGGTSACS-3′	274
nqo12r	5′-VARWGCYTTRAARAAWCGRTGYGT-3′	
nqo8f	5′-GGTTACACCGTTGAATTCTCG-3′	1185
nqo8r	5′-GGATCACCATGGATCACGAAA-3′	
nqo9f	5′-CCGTGAAAATCAAGTACGTGAC-3′	1385
nqo9r	5′-CCATCGTGATGTTCGGCACGA-3′	
nqo13f	5′-CATCTTCGCCTTCACGGT-3′	730
Na^+/H^+r	5′-CACAGACATACGACAAGC-3′	
Na^+/H^+f	5′-TCCAGCGTGCTCGATAAC-3′	531
pcdr	5′-TGCGCACGATGAAGCTCA-3′	
pcdf	5′-TGAGCTTCATCGTGCGCA-3′	548
nqo14r	5′-GATCAGCGCATAGACCTC-3′	

the Portuguese EMBnet Node and the Neural Networks for Promoter Prediction [26]. Comparisons with sequences from other organisms were performed using Blast at NCBI [27] and at the Comprehensive Microbial Resource [28] databases, and multiple sequence alignments were produced according to [29]. Secondary structure analyses were accomplished, using Sosui [30] and Psipred [31].

The complete nucleotide sequences of the *R. marinus* complex I genes were deposited at the EMBL library under the accession numbers: nqo_A-AY972100; nqo_B-AY972822; nqo_8-AY972098; nqo_9-AY972099.

2.3. RT-PCR experiments

To show that the *nhaD* and the *pcd* genes are co-transcribed with the genes nqo_{13} and nqo_{14}, specific forward and reverse oligonucleotides were designed according to the *R. marinus* nqo_B DNA sequence (Fig. 3; Table 1). The DNA amplification conditions for each pair of oligonucleotides were optimised. The total RNA was isolated from cells of *R. marinus*, grown up to a late exponential phase, using RNAeasy Mini and Midi Kits (Qiagen), and treated with DNase I RNase-free (Roche Applied Science). After confirming the absence of DNA contamination, the Reverse Transcriptase (RT)-PCR reactions were performed using the One-step RT-PCR kit (Roche), 70 ng of *R. marinus* RNA and 30 pmol of primer, in a final volume of 50 μl.

2.4. N-terminal sequencing

For amino acid sequencing, NDH-1 was purified as in [19] and its subunits were resolved by tricine SDS-PAGE [32] with 10% T/3% C. Western blot was performed to a polyvinylidene membrane. Upon colouring with Coomassie brilliant blue R, each individual band was cut and submitted to automate Edman degradation [33], and analysed in an Applied Biosystem model 491HT sequencer.

3. Results and discussion

3.1. Analysis of nqo DNA sequences

The analysis of the DNA sequence obtained from the phage isolated with the nqo_1 probe revealed that a 9101-bp gene cluster, nqo_A, contains the open reading frames (ORF) of seven subunits from the *R. marinus* Nqo, namely Nqo_1 to Nqo_7. In addition, nqo_A is flanked upstream by the gene coding for a protein homologous to adenylosuccinate lyase, and downstream, and in the opposite direction, by the open reading frame of malate synthase, both genes not related to NDH-1. Downstream nqo_2 and nqo_1 (Fig. 1), several unknown reading frames (URF), having 1701 and 446 bp, respectively, were identified. These URFs have the same relative position of those reported for the *Paracoccus denitrificans* [34] and the *Rhodobacter capsulatus* [35] NDH-1 operons; however, no sequence similarity is observed between the URFs of the three organisms. RT-PCR experiments using *R. marinus* total RNA have shown that these DNA regions are co-transcribed with the genes nqo_1, nqo_2, and nqo_3 (data not shown). The putative promoter region located −74 to −25 nucleotides upstream the start codon of nqo_7, and the putative sequence for a strong terminator located at +27 to +50 nucleotides downstream from nqo_3, suggest that nqo_7 is the first of a gene cluster that ends at nqo_3 (Fig. 1).

The DNA sequence analysis of an 8086 bp fragment of the phage isolated with the nqo_{12} probe, named nqo_B, revealed that it contains the genes coding for Nqo_{10} to Nqo_{14}. In addition to the Nqo subunits, there are two ORFs, located between nqo_{13} and nqo_{14}, coding for a NhaD (see Section 3.5) and a PCD homologous proteins (Fig. 2). The putative promoter region is predicted at −87 to −42 nucleotides upstream of the start codon of nqo_{10}, suggesting that nqo_{10} is the first gene of the cluster. A DNA sequence resembling a strong terminator is also observed at +24 to +52 nucleotides downstream from nqo_{14} (Fig. 1). It is worth

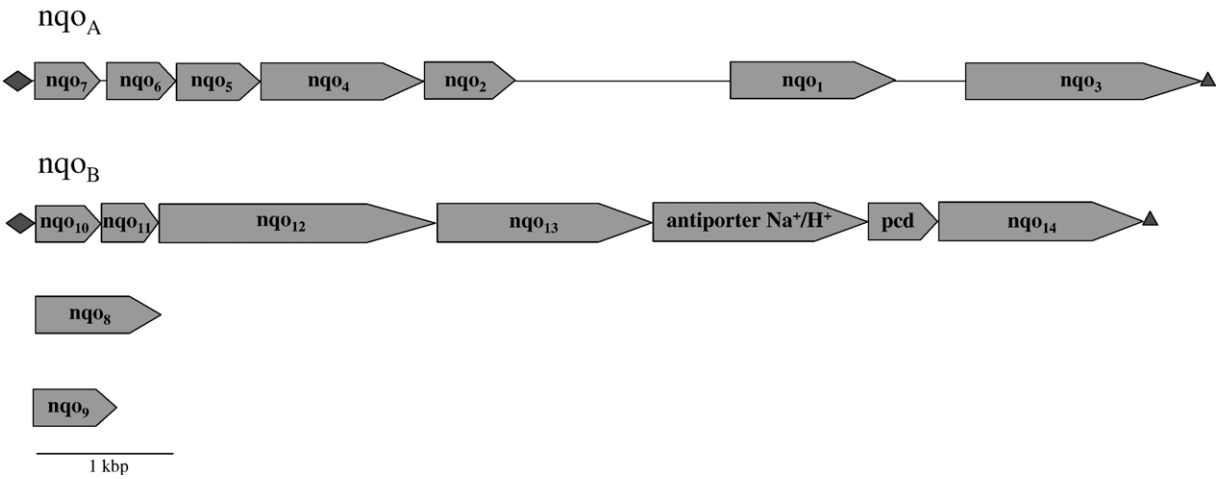
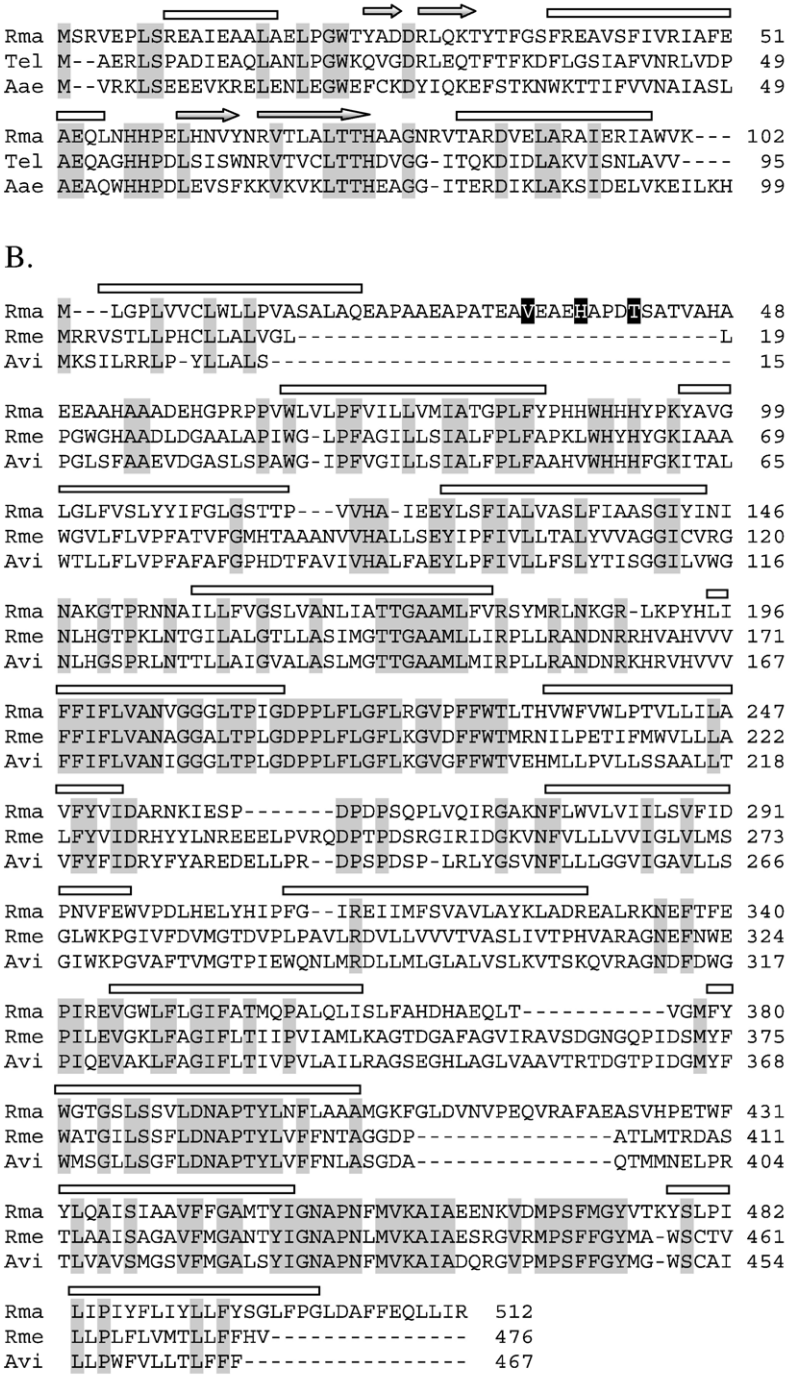


Fig. 1. Genomic organization of *R. marinus* NDH-1. ♦, promoter; ▲, terminator; − unknown reading frames.

A.



C.

PCD				Antiporter			
		Tel	Aae			Rme	Avi
Rma	Identity	43%	30%	Rma	Identity	33%	33%
	Similarity	59%	51%		Similarity	48%	48%
Tel	Identity		39%	Rme	Identity		61%
	Similarity		59%		Similarity		77%

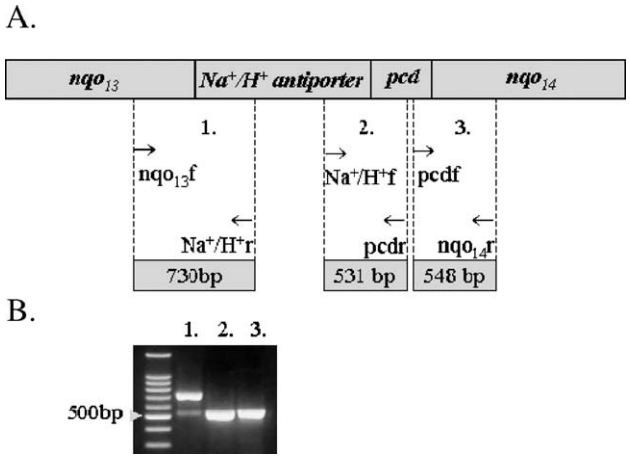


Fig. 3. The *R. marinus* putative PCD and NhaD encoding genes are co-transcribed with the NDH-1 genes nqo_{13} and nqo_{14}. (A) RT-PCR strategy; the expected sizes of the amplified products, if the above-mentioned genes are co-transcribed, are depicted in grey boxes; the arrows represent both the direction of synthesis and its starting point for each primer. (B) Agarose gel electrophoresis of the RT-PCR products; lanes 1, 2, and 3 show the products of the reactions with the same number; 100 bp ladder was used as standard. The smaller and lighter product observed in lane 1 was also present in the PCR reaction carried out as control, therefore being attributed to the unspecific hybridisation of one of the primers.

mentioning that genes coding for proteins which are not related to NDH-1 flank nqoB. Upstream nqo_{10}, there is a gene coding for an exopolyphosphatase homologous protein; downstream nqo_{14}, and in the opposite direction, there is a gene encoding a putative spore maturation protein. Aiming to establish whether the putative *R. marinus* NhaD and PCD encoding genes were co-transcribed along with their upstream and downstream genes, namely nqo_{13} and nqo_{14}, respectively, RT-PCR experiments were performed, using *R. marinus* total RNA and specific oligonucleotides (Table 1, Fig. 3A). The sizes of the DNA products obtained in the RT-PCR reactions are in agreement with those expected if the four genes were co-transcribed (Table 1, Fig. 3B), showing that they are part of a single transcriptional unit.

Southern blot analysis of *R. marinus* genomic DNA digested with either *Bam*HI, or *Not*I, or with the two endonucleases was performed, and the membranes were hybridised with nqo_1 (nqo_A) and nqo_{12} (nqo_B) probes. In all cases, a single band was observed, indicating that a single copy of nqo_1 and nqo_{12} is present in the genome of *R. marinus*.

The genes encoding the membrane arm subunit nqo_8, and the peripheral iron–sulphur centre containing nqo_9, in *R. marinus* PRQ62B genome, have 972 bp and 690 bp, respectively.

3.2. Primary structure analyses of nqo encoded proteins

The primary structures deduced from nqo_A and nqo_B gene clusters and from nqo_8 and nqo_9 present high degree of similarity and identity to their bacterial counterparts. After performing N-terminal sequencing of some subunits of the purified *R. marinus* NDH-1, it was possible to assign five N-terminal sequences to the primary structures of Nqo_1 to Nqo_5 (Table 2). Nqo_1 is the catalytic subunit of complex I, containing the binding-sites for the substrate, NADH, and its cofactor, FMN. Although the motives to bind NADH and FMN in Nqo_1 do not obey Wierenga's rule [36], they are strictly conserved among Nqo_1 homologues [6] and the *R. marinus* subunit is not an exception (Table 3). Residues corresponding to iron–sulphur cluster binding-sites are strictly conserved in the primary sequences of Nqo_1, Nqo_2, Nqo_3, Nqo_6, and Nqo_9, suggesting that *R. marinus* complex I is able to harbour the eight iron–sulphur centres generally assigned to these subunits, namely N_3, N_{1a}, N_4, N_5, N_{1b}, N_2, and N_{6a} and N_{6b} (Table 3) [7]. Since NDH-1 may have several quinone-binding sites [2] (3–4 quinones per complex were determined for *R. marinus* complex I [19]), these sites were searched according to the proposals of Fisher and Rich [37]. In fact, several type I putative quinone-binding sites (aliphatic-$(X)_3$-H-$(X)_{2/3}$-(L/T/S)) are identified in the *R. marinus* NDH-1 subunits (Table 3). There is also a putative quinone-binding site of type I in the amino acid sequence of the *R. marinus* NhaD homologue.

3.3. Secondary structure analyses of the proteins encoded by nqo genes

According to what is generally observed in complex I, subunits Nqo_1 to Nqo_6, and Nqo_9 are predicted to be water soluble, thus forming the peripheral arm of the enzyme. Subunits Nqo_{7-8}, and Nqo_{10-14} are predicted to be transmembranous, in agreement with their location in the membrane arm of the enzyme, folding into fifty-six α-helices, (Table 3). Concerning the putative NhaD, it is predicted to have the twelve transmembrane domains also predicted for its homologues. The PCD homologue is predicted as a water-soluble protein, and to have the same secondary structure pattern observed in its homologues (Fig. 2).

Fig. 2. Comparative alignment of the *R. marinus* putative PCD (A) and NhaD (B) primary structures with homologous sequences from other bacteria. Amino acid residues conserved among the three organisms are shadowed in grey and a putative quinone-binding site is highlighted in black over white characters. The relative position of the secondary structure motives predicted (α-helices—open rectangles; β-sheets—shadowed arrows over the *R. marinus* sequence) for the PCD sequences used in the alignment is strictly conserved. The transmembrane α-helices predicted for the *R. marinus* (Rma) NhaD homologue are signalled above the sequence. Accession numbers: PCD from *Thermosynechococcus elongatus* (Tel)-Q8DHW8, and from *A. aeolicus* (Aae)-O66462; NhaD from *Ralstonia metallidurans* (Rme)-ZP_00022454, and *Azotobacter vinelandii* (Avi)-ZP_0089424. The degree of identity and similarity between the selected sequences is presented (C).

Table 2
Assignment of N-terminal sequences of *R. marinus* NDH-1 subunits

R. marinus subunit	Apparent molecular mass (kDa)	N-terminal sequence
Nqo_1	49	ATNGAQSKAGDWRNYKRVLPP
Nqo_2	25	ADFVKKPVVPLPELH
Nqo_3	60	RITIDGTVYEFEGR
Nqo_4	50	APSLVG

The presence of a gene putatively encoding a PCD, an enzyme that catalyses the regeneration of the 4α-carbinolamine from the biopterin cofactor [38], in nqo_B, led us to think that a prosthetic group resembling a pterin could be harboured by Nqo_3. In fact, similarly to the formate dehydrogenase subunit of formate hydrogenlyases (*e.g. Ralstonia eutropha* [39] and *Eubacterium acidaminophilum* [40]), the Nqo_3 subunit of complex I contains an iron-hydrogenase I-like and a regular formate dehydrogenase modules in a single protein, having the formate dehydrogenases a molybdopternin co-factor. However, the observation that the amino acid sequence of the *R. marinus* complex I subunit is shorter than its counterparts in about 200 amino acid residues, and the fact that it was tried to model the 343 C-terminal amino acid residues of *R. marinus* Nqo_3 with the molybdopterin containing enzymes group H formate dehydrogenases (e.g., *E. coli* [41]), nitrate reductases (e.g., *Desulfovibrio desulfuricans* [42]), and to DMSO reductases (e.g., *R. capsulatus* [43]), and no model was obtained in the region involving the molybdopterin, make the above suggested hypothesis very unlikely.

3.4. Proton pathway-related amino acid residues in *R. marinus* Nqo subunits

Beyond NADH oxidation, NDH-1 also accomplishes an ion-translocation activity across the membranes, whose mechanism is still not understood. Nevertheless, several amino acid residues have been proposed to carry out an important role in this function of the enzyme, and therefore, the *R. marinus* sequences were searched for their presence.

The *R. marinus* Nqo_6 subunit was compared with its bacterial homologous subunits, and searched for the tyrosine residues at positions 114 and 139 (*E. coli* numbering), which are presumably involved in a proton pathway near cluster N2 [44]. The tyrosil 139 is strictly conserved among all the proteins. In contrast, tyrosil 114 is not conserved in *R. marinus* Nqo_6, where a tryptophan residue replaces it. This feature is also observed in the corresponding subunits of the *T. thermophilus* and *A. aeolicus* enzymes.

Subunit Nqo_7 is predicted to have three primary transmembrane helices, suggesting the anchoring of Nqo_7 to the cytoplasmic membrane, hence being part of the membrane domain of NDH-1, as observed in complex I of other organisms. The predicted topology of Nqo_7 is similar to that proposed for most of its homologues, namely *P. denitrificans* [45], with the N-terminal region facing the cytoplasm while its C-terminus is exposed to the periplasmic space. Analysis of the helical wheel prediction for the second transmembrane domain of *R. marinus* Nqo_7 revealed that the conserved acidic amino acid residues Asp71 and Glu73 are placed in opposite sides of the helical column, an observation that is extended to several homologous subunits

Table 3
Features of *R. marinus* complex I subunits

T. thermophilus/E. coli homologues	Mm (kDa)	Tm	Fe–S cluster type	FV	QBM
Nqo_1/NuoF	49.3	–	$[4Fe-4S]^{2+/1+}$ (N3)	NADH FMN	–
Nqo_2/NuoE	25.0	–	$[2Fe-2S]^{2+/1+}$ (N1a)	–	$G^{162}-X_3-H^{166}-X_2-T^{169}$
Nqo_3/NuoG	63.4	–	$[2Fe-2S]^{2+/1+}$ (N1b) $2[4Fe-4S]^{2+/1+}$ (N5, N4)	–	$V^{93}-X_3-H^{97}-X_2-S^{100}$ $I^{184}-X_3-H^{188}-X_2-T^{191}$
Nqo_4/NuoD	50.6	–	–	–	$L^{30}-X_3-H^{34}-X_2-L^{37}$ $I^{61}-X_3-H^{65}-X_2-T^{68}$ $L^{90}-X_3-H^{94}-X_2-L^{97}$
Nqo_5/NuoC	26.6	–	–	–	–
Nqo_6/NuoB	21.6	–	$[4Fe-4S]^{2+/1+}$ (N2)	–	–
Nqo_7/NuoA	13.8	3	–	–	$I^{28}-X_3-H^{32}-X_3-L^{36}$
Nqo_8/NuoH	37.4	8	–		$I^{183}-X_3-H^{187}-X_2-T^{190}$ $V^{221}-X_3-H^{225}-X_3-S^{229}$
Nqo_9/NuoI	27.1	–	$2[4Fe-4S]^{2+/1+}$ (N6a,b)	–	–
Nqo_{10}/NuoJ	19.8	5	–	–	–
Nqo_{11}/NuoK	14.3	3	–	–	–
Nqo_{12}/NuoL	72.8	13	–	–	$G^{101}-X_3-H^{105}-X_2-S^{108}$ $A^{253}-X_2-H^{256}-X_2-T^{259}$ $V^{332}-X_3-H^{336}-X_2-T^{339}$
Nqo_{13}/NuoM	59.5	12	–	–	$L^{233}-X_3-H^{237}-X_2-L^{240}$ $A^{414}-X_3-H^{418}-X_2-L^{421}$
Nqo_{14}/NuoN	54.2	12	–	–	$A^{226}-X_3-H^{230}-X_2-T^{233}$

Sosui's predicted transmembrane helices (Tm); similar predictions were obtained using Psipred. QBM—quinone-binding motif, FV—flavin-binding motif.

Table 4
Relationship between antiporter-related amino acid sequences

	(%)	NhaD members	Nqo_{12} members	Nqo_{13} members	Nqo_{14} members	MrpA members	MrpD members
R. marinus NhaD	I	29	8	8	8	8	8
	S	44	19	22	25	18	28
Within each group	I	29	25	20	20	31	34
	S	44	43	44	38	50	56

The primary structure of *R. marinus* NhaD was compared with homologous sequences of NhaD antiporters, Nqo_{12}, Nqo_{13} and Nqo_{14} subunits of complex I, and with MrpA and MrpD subunits of Mrp antiporters using CLUSTAL W. Percents of identity (I) and similarity (S) are presented.

of different organisms. For instance, the homologous subunit from *Homo sapiens*, whose sequence displays only 11% identity but 29% similarity to the *R. marinus* counterpart, and that contains a unique primary transmembrane α-helix, shows the conserved glutamate and aspartate residues located in opposite sides of the helical column, thus reinforcing the proposal that these residues may play an important functional role in the activity carried out by complex I [45]. Recently, Kao and colleagues have shown that E81 from the *E. coli* homologous subunit, NuoA, is important for NDH-1 activity [46]. There are five other strictly conserved acidic residues in complex I subunits, which are suggested to be involved in proton translocation by Nqo: E37 and E73 from Nqo_{11} [47], E146 from Nqo_{12}, E141 in Nqo_{13} and E134 from Nqo_{14} (*P. denitrificans* numbering) [7]. All these residues are also conserved in the *R. marinus* counterparts. However, it should be stressed that the presence of protonable amino acid residues is not a demand for proton translocation, as observed in the case of other proton translocating enzymes, such as oxygen reductases [48,49].

3.5. The R. marinus Na^+/H^+ antiporter

As stated above, one of the ORFs encoded by nqo_B was assigned as a homologue of NhaD Na^+/H^+ type antiporters, since, when its deduced amino acid sequence was compared against sequence databases, the only significant hits were NhaD proteins. Considering that the membrane arm of complex I contains four Na^+/H^+ antiporter related subunits (Nqo_{11-14}) [11], the amino acid sequence of the *R. marinus* NhaD was also compared to Nqo_{12}, Nqo_{13}, and Nqo_{14}, and to their homologues MrpA and MrpD subunits of Mrp Na^+/H^+ antiporters (Table 4). The putative NhaD of *R. marinus* displays 29% identity and 44% similarity with other NhaD antiporters, and only 8% identity and 18 to 28% similarity with members of the other groups. Therefore, it can be concluded that the *R. marinus* NhaD is not just another copy of the Mrp-like subunits, but indeed a homologue of a NhaD Na^+/H^+ antiporter.

4. Concluding remarks

Based on the above results, genetic evidence for the presence of a type I NADH:quinone oxidoreductase in the respiratory chain of the microaerophilic bacterium *R. marinus* was provided, adding this complex to the small number of bacterial complexes I so far described. Although the genomic organisation of its encoding genes in two putative operons and two independent genes is not unusual, the presence of *pcd* and *nhaD* putative genes among *nqo* genes is unique. Furthermore, it was clearly shown that the URFs found in nqo_A, and the accessory genes in nqo_B form single transcriptional units with the canonical flanking *nqo* genes.

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