



Functional role of the MrpA- and MrpD-homologous protein subunits in enzyme complexes evolutionary related to respiratory chain complex I[☆]

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ABSTRACT

NADH:quinone oxidoreductase or complex I is a large membrane bound enzyme complex that has evolved from the combination of smaller functional building blocks. Intermediate size enzyme complexes exist in nature that comprise some, but not all of the protein subunits in full size 14-subunit complex I. The membrane spanning complex I subunits NuoL, NuoM and NuoN are homologous to each other and to two proteins from one particular class of Na⁺/H⁺ antiporters, denoted MrpA and MrpD. In complex I, these ion transporter protein subunits are prime candidates for harboring important parts of the proton pumping machinery. Using a model system, consisting of *Bacillus subtilis* MrpA and MrpD deletion strains and a low copy expression plasmid, it was recently demonstrated that NuoN can rescue the strain deleted for MrpD but not that deleted for MrpA, whereas the opposite tendency was seen for NuoL. This demonstrated that the MrpA-type and MrpD-type proteins have unique functional specializations. In this work, the corresponding antiporter-like protein subunits from the smaller enzymes evolutionarily related to complex I were tested in the same model system. The subunits from 11-subunit complex I from *Bacillus cereus* behaved essentially as those from full size complex I, corroborating that this enzyme should be regarded as a *bona fide* complex I. The hydrogenase-3 and hydrogenase-4 antiporter-like proteins on the other hand, could substitute equally well for MrpA or MrpD at pH 7.4, suggesting that these enzymes have intermediate forms of the antiporter-like proteins, which seemingly lack the functional specificity.

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1. Introduction

NADH:quinone oxidoreductase or complex I (EC 1.6.99.3) is the largest enzyme in the respiratory chain of mitochondria and bacteria. The mammalian enzyme comprises 45 protein subunits, but of these, fourteen protein subunits make up a conserved core structure also found in bacteria. Seven subunits protrude from the membrane and contain a flavin prosthetic group and eight iron-sulfur clusters that guide electrons from the oxidation of NADH towards the quinone binding site [1]. The remaining seven subunits make up the membrane domain of the enzyme complex. These proteins are lacking prosthetic

groups, but nevertheless, this domain must harbor important parts of the energy coupling machinery. The structure of the soluble domain of complex I has been solved to 3.1 Å resolution [2,3], whereas recently, the membrane spanning domain has also been revealed to about 3.0 Å [4,5]. Already in structures solved at lower resolution, the three homologous proteins NuoL, NuoM and NuoN stood out as three virtually identical structural entities, except for a NuoL C-terminal extension in the form of a membrane-parallel helix extending from the distally located NuoL subunit towards the proximal NuoN [6,7].

The complex I protein subunits can be divided into functional modules [1]. This reflects that the evolution of the large enzyme complex has occurred from smaller functional building blocks [8]. The NADH dehydrogenase or N-module is composed of the NuoE, NuoF, and NuoG protein subunits, and can be regarded as an electron input device. The Q-module enzyme comprises the NuoB, NuoC, NuoD, and NuoI protein subunits, where NuoB and D resemble the small and large subunit of NiFe hydrogenase [9]. The metal site in the hydrogenase corresponds to a quinone-binding site in complex I [10], explaining the module name. Finally the P-module, for proton pumping, is made up of all the hydrophobic proteins NuoA, NuoH, NuoJ, NuoK, NuoL, NuoM, and NuoN [1]. The three large proteins NuoL, NuoM and NuoN show primary

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sequence similarity to two protein subunits in the Mrp Na^+/H^+ antiporter complex, MrpA and MrpD [8,11]. Therefore, these proteins come out as main suspects in providing ion channels for the proton pumping machinery. Such putative proton pathways have also been visualized in the structure [4]. The Mrp antiporter complex is composed of seven proteins in total, including MrpA and MrpD. It has been demonstrated that all the seven Mrp-complex protein subunits must be present to have a functional Na^+/H^+ antiporter [12,13].

Complex I homologous protein assemblies are also found in several different types of smaller enzyme complexes containing fewer units than the 14 proteins in the “standard” bacterial enzyme that are also conserved in the mitochondrial complex I. One such smaller enzyme is predicted to be a *bona fide* complex I enzyme, lacking the N-module ([14], Table 1). This enzyme is the putative ancestor of all present day complex I and could probably interact with different electron donors/acceptors [15], whereas the 14-subunit enzymes operate solely with NADH/NAD⁺. Other homologous smaller enzyme complexes are membrane-spanning hydrogenases that are typically defined by the presence of the NiFe active site cysteine ligands in the NuoD homologous subunit. These membrane-bound hydrogenases come in several varieties. Hydrogenase-4 contain three MrpA- and MrpD-homologous proteins, i.e. three putative ion channels, whereas hydrogenase-3 have only one such subunit (Table 1). Both these hydrogenase varieties exist in *Escherichia coli*. The smaller hydrogenase-3 enzyme, with protein subunits denoted Hyc [16], is functioning together with FdhH, a formate dehydrogenase protein for which the structure was solved already in 1997 [17]. The hydrogenase-3 enzyme was shown to be the main hydrogen producing enzyme in *E. coli* whereas the soluble hydrogenase-1 and -2 enzymes were responsible for respiratory hydrogen oxidation [18]. The hydrogenase-4 in *E. coli* is encoded by the *hyf* operon and was postulated to also operate with FdhH and carry out energy conservation by performing formate dependent proton translocation [19]. The Hyf enzyme complex has never been biochemically characterized. The *hyf* operon was found to be silent in wild type *E. coli* but its expression could be activated [20]. It was also demonstrated that *hyf*, in spite of activation, cannot functionally substitute for the loss of *hyc*. A CO-induced hydrogenase has been described in

Rhodospirillum rubrum, which generates energy from the oxidation of CO to CO₂ coupled to the production of H₂ [21]. The Ech hydrogenase was first described in *Methanosarcina barkeri* [22] and has since been found in several methanogenic archaea [9]. Other archaeal hydrogenases, denoted Mbh and Mbx, were first described in the hyperthermophile *Pyrococcus furiosus* [23–25]. Their evolution and relation to complex I were recently reviewed in [26].

By understanding more about the building blocks and their primordial function, important clues to the molecular function of present day complex I can be deduced (see Fig. 1). A survey of the distribution of different types of complex I in the tree of life recently showed that a compact 11-subunit complex I was found both in the archaeal and the eubacterial kingdoms, whereas the full size, 14-subunit complex I was only found in some eubacterial phyla [14]. This demonstrated that such an 11-subunit complex I was the last common ancestor of all present day complex I enzymes. Membrane-bound hydrogenases have also been suggested to be ancestors of complex I [8,27,28]. Indeed, a membrane bound hydrogenase is a likely evolutionary progenitor of complex I but this enzyme must have differed from the present day membrane-bound hydrogenases in several respects [15,29]. The antiporter-like ion transporter proteins in membrane-bound hydrogenases were lacking primary sequence features that were present in both the *bona fide* antiporter proteins MrpA and MrpD and in the complex I proteins NuoL, NuoM and NuoN, and therefore must have evolved separately in hydrogenases, and only after the split from the last common ancestor [29].

A model system, where the function of the Mrp-type ion transporter proteins can be assessed, was recently developed. Deletion of *mrpA* or *mrpD* from the *Bacillus subtilis* chromosome resulted in Na^+ and pH sensitive growth phenotype, which could be explored in complementation tests. The properties of the complex I subunits NuoL, NuoM and NuoN from *E. coli* complex I were investigated using the model system. NuoL from *E. coli* expressed in the *B. subtilis* $\Delta mrpA$ brought back the wild type growth properties, whereas expression of *E. coli* NuoN in the

Table 1
List of homologous subunits.

<i>E. coli</i> complex I	<i>B. cereus</i> complex I	<i>E. coli</i> FHL-1 (Hyc)	<i>E. coli</i> FHL-2 (Hyf)	<i>R. rubrum</i> CO-induced hydrogenase	<i>M. barkerii</i> Ech hydrogenase	<i>B. subtilis</i> Mrp antiporter
<i>N module</i>						
NuoE	–					
NuoF	–					
NuoG	–					
<i>Q module</i>						
NuoB	NuoB	HycG	HyfI	CooL	EchC	
NuoC ¹	NuoC	HycE (N-ter)	HyfG(N- ter)	CooU	EchD	
NuoD ¹	NuoD	HycE (C-ter)	HyfG(C- ter)	CooH	EchE	
NuoI	NuoI	HycF	HyfH	CooX	EchF	
<i>P module</i>						
NuoA	NuoA	–	–	–	–	MrpA
NuoH	NuoH	HycD	HyfC	CooK	EchB	C-term
NuoJ	NuoJ	–	–	–	–	MrpC
NuoK	NuoK	–	HyfE	–	–	MrpA/D
NuoL/M/N	NuoL/M/N	HycC	HyfB/D/F	CooM ²	EchA	

¹ In *E. coli*, NuoC and NuoD are in fact also fused into one subunit [41], just as in HycE and HyfG, but this is not a general feature of complex I.

² The CooM protein comprises two antiporter-like polypeptides fused into one polypeptide.

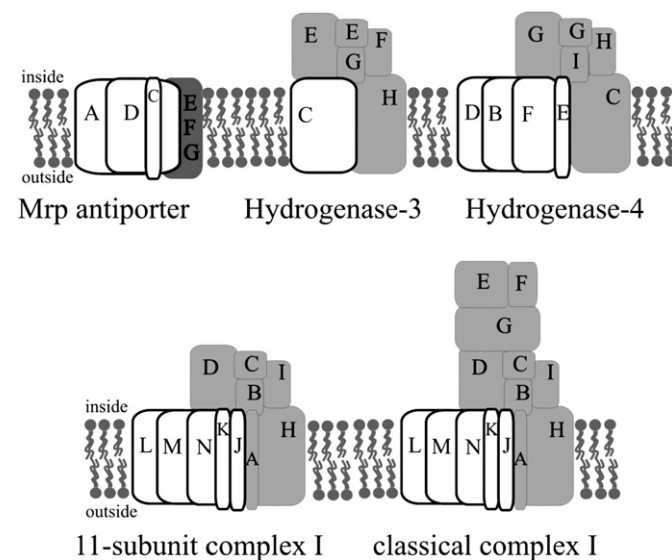


Fig. 1. Schematic representation of the protein subunits in the respective enzyme complexes. Subunit nomenclature is also summarized in Table 1. The *bona fide* Mrp-antiporter contains two homologous subunits, MrpA and MrpD. The MrpEFG subunits are not related to any present-day complex I proteins. Hydrogenase-3 and hydrogenase-4 contain one or three homologous proteins of the MrpA/D type respectively. In addition, both types of hydrogenases interact with a formate dehydrogenase unit in *E. coli* (not shown). The membrane-spanning part of 11-subunit complex I and classical 14-subunit complex I is fully conserved and contains three MrpA/D type proteins. In some complex I enzymes, such as that from *E. coli*, the NuoC and NuoD homologues are fused into one subunit, just as in the corresponding hydrogenase subunits HycE and HyfG ([41] see also Table 1).

same deletion strain did not improve the growth at all. The opposite was true for the *B. subtilis* $\Delta mrpD$ strain, that was efficiently complemented by NuoN, and to a much lesser extent by NuoL [30]. It was also noticed that the complementation observed at pH 7.4 did not require Mrp-complex formation but merely the presence of all subunit types. At pH 8.4, where no complementation with complex I subunits was seen, Mrp-complex formation was shown to be necessary for function [30]. The simplest explanation for the observed growth properties was that each protein comprised a single ion channel rather than a complete antiporter. Since Na^+ translocation had previously been observed in NuoL [31] MrpA and NuoL were tentatively assigned as Na^+ channels whereas MrpD and NuoN were proposed to be H^+ channels. In this work we have addressed the issue of ion-specificity further, by measuring the internal sodium concentration in cells by $^{23}\text{Na}^+$ -NMR.

The compact, 11-subunit complex I is not present in *B. subtilis*, but is found in the close relative *Bacillus cereus*. Membrane-bound hydrogenases can be of several types. Examples of antiporter-like proteins are HycC from hydrogenase-3, HyfB, HyfD and Hyf from hydrogenase-4 [19,32], CooM from CO-induced hydrogenase, actually comprising two subunits fused into one [21] and EchA from Ech-hydrogenase [33], see Fig. 1 and Table 1. In this work, the previous phylogenetic analyses [29] were repeated, since the number of available sequences had increased significantly since the completion of that work in 2002. Subsequently, the *B. subtilis* model system was used to investigate the ion translocation abilities of the individual NuoL, NuoM and NuoN complex I subunits from 11-subunit complex I from *B. cereus*, to elucidate if the functional properties are indeed conserved. In addition, the HycC protein subunit from *E. coli* hydrogenase-3 and the HyfB, HyfD and HyfF subunits from *E. coli* hydrogenase-4 were cloned and expressed in the model system, to investigate if the theoretical predictions regarding their functionality could be verified experimentally.

2. Materials and methods

2.1. Construction of the phylogenetic tree of ion-transporter subunits from complex I and their homologues in membrane-bound hydrogenases

Protein primary sequences of HycC subunit of hydrogenase-3, HyfB, HyfD, HyfF subunits from hydrogenase-4, EchA subunit from Ech hydrogenase, and NuoL, NuoM and NuoN subunit of 11 subunit, 14 subunit, and 12 subunit complex I enzymes from both Archaea and Eubacteria were collected from TIGR and NCBI and aligned using Clustal W with default settings. The aligned dataset was analyzed, edited and converted into MEGA format by using Data Analysis in Molecular Biology and Evolution, DAMBE ver 4.13. Unrooted phylogenetic trees were created using MEGA version 4.0 with boot-strap support of 100 replicates in the Neighbor-Joining method. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Poisson correction method and are in the units of the number of amino acid substitutions per site. All positions containing gaps and missing data were eliminated from the dataset (complete deletion option). For substitutions in the dataset the included option was used for the whole sample. Pattern among lineage was set to homogeneous and rates among site were set to uniform rates. Phylogenetic analyses were conducted in MEGA4. Protein accession numbers are listed in the supplementary material.

2.2. $^{23}\text{Na}^+$ -NMR experiments

The $^{23}\text{Na}^+$ -NMR experiments were done essentially as in [34]. Briefly, the cells were grown without extra Na^+ added to the media to OD 0.5, and then challenged with 80 mM NaCl for 1–2 h. After that, the cells were harvested, washed with Na^+ -free buffer and resuspended at 0.32 g cells/ml buffer. To 400 μl of the cell suspension D_2O and the shift reagent $\text{Tm}(\text{DOTP})_5^-$ were added to concentrations 10% and

0.8 mM, respectively. At least 512 scans were accumulated for each spectrum, and the internal and external Na^+ concentrations were calculated from the signal intensities. The internal signal was multiplied by 2.5, to compensate for the extreme broadening of the cell interior (see discussion in [34]).

2.3. Cloning and construction of expression plasmids

Bacterial strains, plasmids and primers used are listed in Table 2. *E. coli* cells and *B. subtilis* cells were routinely grown aerobically at 37 °C and 200 rpm in LB medium as before. For solid media, 1.5% agar was added. *B. subtilis* strains were grown aerobically at 37°C and 200 rpm in nutrient sporulation medium with phosphate (NSMP [35]) and were kept on Tryptone Blood Agar Base (TBAB) plates. Antibiotics were added in the following concentrations when appropriate: 100 $\mu\text{g}/\text{ml}$ ampicillin, 12.5 $\mu\text{g}/\text{ml}$ chloramphenicol and 10 $\mu\text{g}/\text{ml}$ kanamycin for *E. coli*, 5 $\mu\text{g}/\text{ml}$ chloramphenicol and 2 $\mu\text{g}/\text{ml}$ phleomycin for *B. subtilis*. All standard molecular biological techniques were done as before [30]. Primers (Table 2) were synthesized by MWG Biotech AB, Germany and Fermentas Life Sciences. PCR products were purified from agarose gel with the Jetsorb gel extraction kit (Genomed) and restriction enzyme digested vectors and DNA fragments were purified by DNA Clean Up (Genomed) or Jetsorb. Chromosomal DNA from *B. cereus* was prepared as described by Marmur [36], and for *E. coli* chromosomal DNA the method of Wilson [37] or the Gene Jet™ Genomic DNA Purification Kit (Fermentas) was used. DNA sequencing reactions were carried out using Big Dye™ (Applied Biosystems) and the subsequent sequence analysis was performed at the Biomolecular Resource Facility, Lund University.

B. cereus chromosomal DNA was used as a template for amplification of *nuoL*, *nuoM* and *nuoN*, whereas *E. coli* chromosomal DNA was used for amplification of *hycC*, *hyfB*, *hyfD* and *hyfF* using the primers shown in Table 2. In the upstream primer, a unique restriction-enzyme recognition site was created, for *nuoL* a *HindIII* site and for *nuoM*, and *nuoN* a *XbaI* site. In the downstream primer sequence no restriction-enzyme recognition site was introduced, since a DNA polymerase leaving blunt ends was used. The stop codon was removed from the respective gene sequences, to produce fusion proteins to cytochrome c_{550} when the gene fragments were cloned into pTRC19 [38] and/or pTCH [39]. The constructs were transformed into *E. coli* JM109 [40]. The resulting, correct constructs were named pVM12, pVM13, pVM14, pVM18, pVM19, pVM21 and pVM22 (Table 2). These plasmids were subsequently used as template for the amplification of *nuoLcyc*, *nuoMcyt*, *nuoNcyt*, *hycCcyt*, *hyfBcyt*, *hyfDcyt* and *hyfFcyt* respectively, for subcloning of the cytochrome-tagged polypeptides into the shuttle vector pCW6. In the downstream primers, a unique *PstI* site was introduced, except in *nuoMcyt* that contains an internal *PstI* site, and therefore *Sall* was used instead. In the upstream primers a *XbaI* site was created in all the constructs (Table 2). The pCW6 was purified from *E. coli* GM3819 to obtain unmethylated plasmid DNA that can be digested with *XbaI*. After confirming the correct constructs by DNA sequencing, the resulting constructs were named pVM15, pVM16, pVM17, pVM20, pVM23, pVM24 and pVM25.

2.4. *B. subtilis* growth studies

The *B. subtilis* strains were grown essentially as described previously [30]. The same 250 ml baffled E-flasks were used. The flasks have an attached side-arm that allowed convenient measurement of the optical density (OD) of the culture without opening the flasks or changing the growth medium volume throughout the experiment. According to the manufacturer, the NB contains about 7 mM Na^+ when used as recommended (8 g/L). The NaCl concentrations indicated in each experiment always refer to the amount of NaCl added to the growth medium. As before, the *B. subtilis* strains to be studied were taken from the -80°C freezer and incubated on plates with no added NaCl, at 37°C for 8 h,

Table 2

Bacterial strains, plasmids and primers used in this work.

Bacterial strain	Genotype	Reference/Source
<i>B. subtilis</i> 168A	Wild type, <i>trpC2</i>	<i>Bacillus</i> Genetic Stock Center (type train)
<i>B. subtilis</i> $\Delta mrpA$	$\Delta mrpA$ ble ^r	[30]
<i>B. subtilis</i> $\Delta mrpD$	$\Delta mrpD$ ble ^r	[30]
<i>B. cereus</i> ATCC 14579	Wild type	DSMZ (Type strain, DSM31, ATCC 14579)
<i>E. coli</i> JM109	<i>endA1 glnV44 thi-1 relA1 gyrA96 recA1 mcrB⁺ Δ(lac-proAB) e14-[F' traD36 pro AB⁺ lac^f lacZΔM15]</i> <i>hsdR17(r_k[−] m_k⁺)</i>	[40]
<i>E. coli</i> XL1-Blue	<i>recA1, endA1, gyrA96, thi, hsdR17, supE44, relA1 (lac)</i>	Promega
<i>E. coli</i> GM3819 (Dam [−])	<i>dam-16::Kan thr-1 leuB6 thi-1 argE3 hisG4 proA2 lacY1 galK2 mtl-1 xyl-5 ara-14 rpsL31 tsx-33</i> <i>supE44 rfbD1 kdgK51</i>	
Plasmids	Relevant properties	
pCW6	Cm ^r	Claes von Wachenfeldt
pVM6	<i>mrpDcytc</i> Cm ^r	[38]
pVM11	<i>mrpAcytc</i> Cm ^r	[38]
pVM12	<i>nuoL_{Bc}cytc</i> , Amp ^r	This work
pVM13	<i>nuoM_{Bc}cytc</i> , Amp ^r	This work
pVM14	<i>nuoN_{Bc}cytc</i> , Amp ^r	This work
pVM15	<i>nuoL_{Bc}cytc</i> , Cm ^r	This work
pVM16	<i>nuoM_{Bc}cytc</i> , Cm ^r	This work
pVM17	<i>nuoN_{Bc}cytc</i> , Cm ^r	This work
pVM18	<i>hycCcytc</i> , Amp ^r	This work
pVM20	<i>hycCcytc</i> , Cm ^r	This work
pVM19	<i>hyfBcytc</i> Amp ^r	This work
pVM21	<i>hyfDcytc</i> , Amp ^r	This work
pVM22	<i>hyfFcytc</i> , Amp ^r	This work
pVM23	<i>hyfBcytc</i> , Cm ^r	This work
pVM24	<i>hyfDcytc</i> , Cm ^r	This work
pVM25	<i>hyfFcytc</i> , Cm ^r	This work
Primers	Restriction enzyme site	Primer sequence
<i>nuoL_{Bc}_for</i>	<i>HindIII</i>	5' CGTACGAAGCTTCAGGTGGA 3'
<i>nuoL_{Bc}_rev</i>	–	5' TCGTAAATCCCTCCCGTAT 3'
<i>nuoM_{Bc}_for</i>	<i>XbaI</i>	5' GTGGTTTCTCTAGATGTACT 3'
<i>nuoM_{Bc}_rev</i>	–	5' CTCACCCCAATGTTTCA 3'
<i>nuoN_{Bc}_for</i>	<i>XbaI</i>	5' AAACATTGGGGTCTAGAAGG 3'
<i>nuoN_{Bc}_rev</i>	–	5' TTGTACCACGTTCCCTAAGAAGA 3'
<i>hycC_for</i>	<i>XbaI</i>	5' CAGCAAAGCTTAGCGTGAGC 3'
<i>hycC_rev</i>	–	5' GGCTCCTCGTGAACAATAATC 3'
<i>hyfB_for</i>	<i>XbaI</i>	5' GAAAGATCTAGAGCGCTTG 3'
<i>hyfB_rev</i>	–	5' GACGGCAATAGCGATTAGCA 3'
<i>hyfD_for</i>	<i>HindIII</i>	5' CTCATTAAAGCTTGACCTGGCTTG 3'
<i>hyfD_rev</i>	–	5' CTGCAACAGGTCCGGCGGATTAC 3'
<i>hyfF_for</i>	<i>HindIII</i>	5' GTCATTAAAGCTTGTTACTGGC 3'
<i>hyfF_rev</i>	–	5' ACGTTCACCTGTGTTCTCCGA 3'
<i>nuoL_{Bc}_for_pCW6</i>	<i>XbaI</i>	5' CGCTCAAAGTCTAGAATTGTGACCG 3'
<i>nuoM_{Bc}_for_pCW6</i>	<i>XbaI</i>	5' GTCATTATTTGCTCTAGACGGG 3'
<i>nuoN_{Bc}_for_pCW6</i>	<i>XbaI</i>	5' GATGGATATCTAGACGTTATTTAGC 3'
<i>nuoL_{Bc}_rev_pCW6</i>	<i>Sall</i>	5' CCGTACTGTCTGACAATCGTTTA 3'
<i>nuoM_N_rev_pCW6</i>	<i>PstI</i>	5' CCGTACTGTCTGCAGTCGTTTA 3'
<i>hycC_for_pCW6</i>	<i>XbaI</i>	5' CACGGACTCTAGAGATCTCACCTTG 3'
<i>hycC_rev_pCW6</i>	<i>PstI</i>	5' CCGTACTGTCTGCAGTCGTTTA 3'
<i>hyfB_for_pCW6</i>	<i>XbaI</i>	5' CTGGTTTTCTAGAGGCGGGGAGGATC 3'
<i>hyfD_for_pCW6</i>	<i>XbaI</i>	5' CCTTCTGCTCTAGAATGGGTCT 3'
<i>hyfF_for_pCW6</i>	<i>XbaI</i>	5' CCCACGGCACTCTAGACGTG 3'
<i>hyfBDF_rev_pCW6</i>	<i>PstI</i>	5' CCGGTCTGCAGGCAGCAGTC 3'

and were then used immediately to inoculate the liquid cultures. Most of the growth studies were repeated several times, but all experiments were repeated in at least five independent experiments.

The generation time *g* is the time it takes for bacteria to divide. The *g* was calculated using Eqs. (1) and (2), where OD1 and OD2 are optical densities from the logarithmic growth phase, *t*₁ and *t*₂, and *k* is a growth constant.

$$k = \frac{(\ln(OD2) - \ln(OD1))}{(t_2 - t_1)} \quad (1)$$

$$g = \frac{\ln 2}{k} \quad (2)$$

3. Results

3.1. The role of cation transport in the deletion strain phenotype

The MrpA, MrpD, NuoL, NuoM and NuoN are all homologous proteins, but the primary sequence of MrpA is more similar to NuoL, whereas MrpD is grouping with NuoN [29]. This coincided with the recently determined functional similarity of MrpA and NuoL, and MrpD and NuoN [30]. The unambiguous role of Na⁺ in the observed deletion strain growth defect was demonstrated by measuring the internal Na⁺ concentration after a two hour 80 mM sodium challenge using ²³Na⁺-NMR and a shift agent [34]. Assuming that the NMR sample volume consists of 20% cells, the *B. subtilis* $\Delta mrpA$ strain contained 56 ± 17 mM Na⁺ and the *B. subtilis* $\Delta mrpD$ strain contained 57 ±

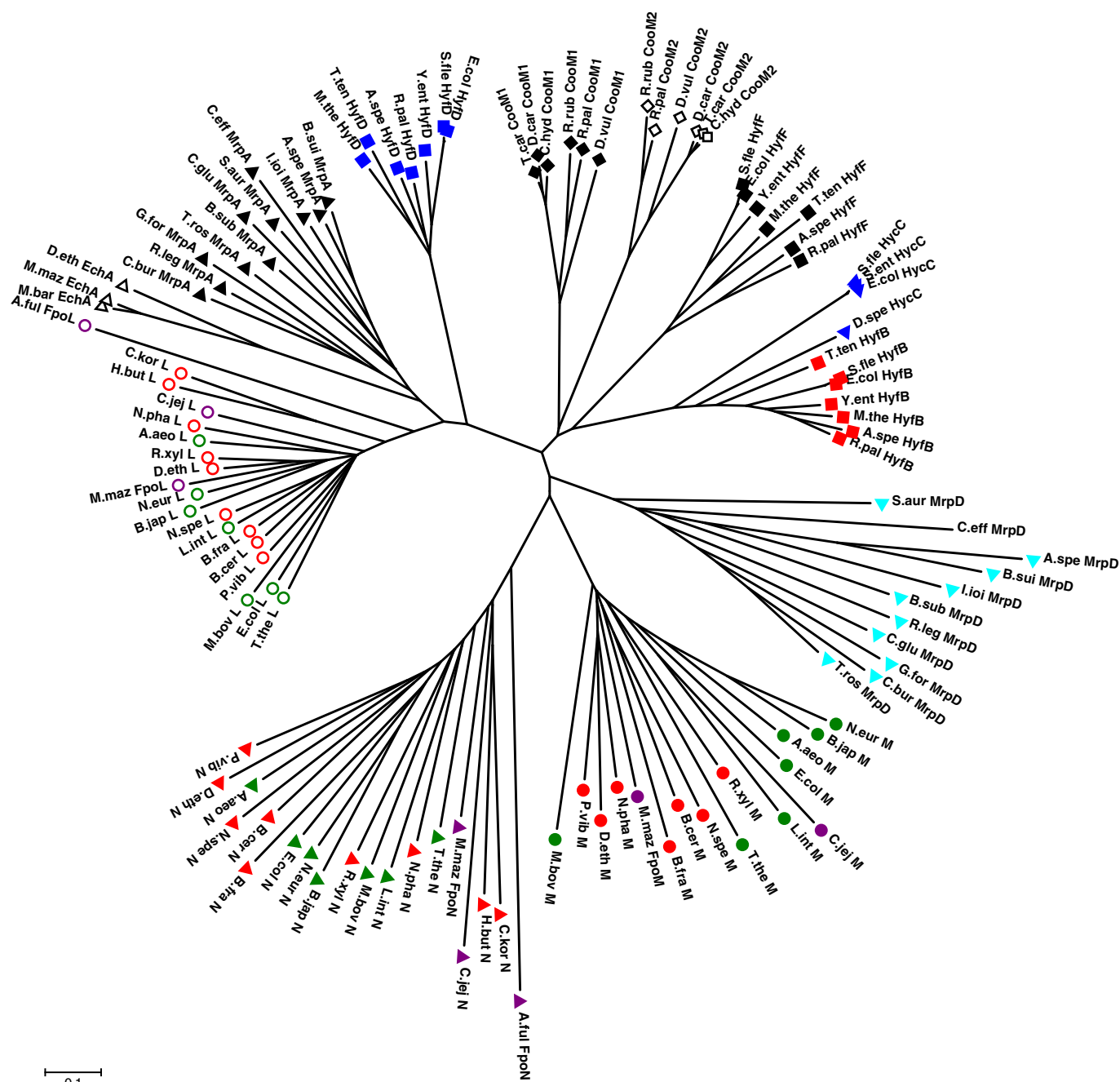


Fig. 2. Phylogenetic tree of the MrpA and MrpD-type proteins comprising Nuol, M and N of complex I and the homologous subunits from membrane-bound hydrogenases. The sample is chosen to represent the most distant branches of the tree of life. Nuol from organisms containing 14-subunit, 12-subunit or 11-subunit complex I is shown in green, violet and empty red circles respectively. Nuom from organisms containing 14-subunit, 12-subunit and 11-subunit complex I is shown in green, violet and red filled circles. Nuon from organisms containing from 14-subunit, 12-subunit and 11-subunit complex I is shown in green, violet and red filled triangles (upward pointing). HycC from hydrogenase-3 is shown with filled blue triangles (downward pointing), and EchA from Ech-hydrogenases with empty downward pointing triangles. The N-terminal part of Coom from carbon monoxide-induced hydrogenase is denoted Coom1 and represented by black filled diamonds whereas the C-terminal part is labeled Coom2 and is shown as empty diamonds. HyfB, HyFD and HyFF subunits from hydrogenase-4 are shown as filled red, blue and black squares respectively. MrpA and MrpD from the Mrp-antiporter are shown as filled (upward pointing) black triangles and filled, light blue (downward pointing) triangles respectively. Full names of organisms and protein sequence accession numbers are given in the supplementary material.

5 mM Na^+ . When MrpA was expressed from a plasmid in the ΔmrpA strain, substantially less Na^+ was retained (19 ± 13 mM), demonstrating that the antiporter is now functional. Expression of MrpA in the ΔmrpD strain, on the other hand, did not alter the intracellular Na^+ content of that strain at all, illustrating that both subunits must be present to have a functional Mrp-antiporter. In this work the analyses were repeated for *B. subtilis* ΔmrpA cells expressing the complex I subunits Nuol and Nuon. As expected, expression of Nuol significantly decreased the internal Na^+ concentration (36 ± 5 mM) whereas

expression of Nuon caused a very small effect, if any (48 ± 8 mM). This corroborates the functional difference of MrpA/Nuol and MrpD/Nuon, as originally predicted from sequence.

3.2. Repeated phylogenetic analyses

As mentioned in the introduction, it was also noted that hydrogenases contained transporter protein subunits with lower sequence conservation, which could not be accurately assigned as MrpA-type or

MrpD-type [29]. These previous results were however derived from a much smaller dataset, and therefore the phylogenetic analyses were repeated with a larger dataset of currently available primary sequences of the proteins in question. A phylogenetic tree, showing representative members of the dataset, is shown in Fig. 2. NuoL, NuoM and NuoN subunits from all types of complex I, including the compact 11-subunit enzyme and hydrogenase subunits from enzymes having just one, two or three different putative ion transporter subunits (Table 1), were compared.

In the unrooted phylogenetic tree (Fig. 2), transporter-protein subunits NuoL, NuoM, and NuoN from all the different types of complex I formed clusters comprising each subunit, corroborating the previous notion that the compact 11-subunit complex I enzymes should indeed be regarded as a *bona fide* complex I [14], in spite of the missing N-module. Just as previously observed, two distinct domains formed in the tree, comprising MrpA-type and MrpD type polypeptides. The 11-subunit complex I subunits did not deviate from this general pattern, and just like the classical complex I subunits, the NuoM and NuoN ended up in the MrpD domain and NuoL in the MrpA domain, predicting that the proteins have the same function.

Among hydrogenase-4 (i.e. Hyf proteins) that have three transporter subunits just like complex I, HyfB is consistently the longer polypeptide, suggesting that it originated from NuoL, but in the phylogenetic tree HyfB sequences are ending up in an intermediate position, close to the HycC cluster. The HyfD sequence on the other hand, is grouping closer to NuoL on the MrpA-type side. Hydrogenase-3 enzymes (i.e. Ech and Hyc) that have only one transporter subunit still form separate groups, not related to the type of hydrogenase. The EchA from the methanogens forms a group closer to NuoL in the MrpA-type cluster, whereas the HycC polypeptides are found together with HyfB and HyfF. Taken together, the new results seem to corroborate the previous findings that the transporter subunits of hydrogenases are separately evolved forms, presumably with less functional specificity than the *bona fide* Mrp antiporter subunits and the *bona fide* complex I subunits.

3.3. Complementation of *B. subtilis* $\Delta mrpA$ and $\Delta mrpD$ strains with 11-subunit complex I ion transporter proteins

Subsequently, the predictions were tested experimentally. The *B. subtilis* Mrp deletion strains, previously developed as a model system to study the function of the homologous ion transporter subunits from *E. coli* complex I [30], were implemented to investigate the function of the ion-transporter proteins from the 11-subunit complex I from *B. cereus*. To be able to monitor and compare the amounts of protein produced, the genes encoding for NuoL, NuoM and NuoN subunits were tagged with cytochrome c as before [38]. The cytochrome tagged fusion constructs were subsequently sub-cloned to a low copy number plasmid, containing the IPTG inducible P_{spac} promoter, thereby producing low, physiologically relevant amounts of proteins in bacteria, as was previously done for the *E. coli* NuoL, NuoM and NuoN subunits.

The *B. cereus* complex I subunits were restoring the growth of *B. subtilis* deletion strains in a similar way as the corresponding *E. coli* complex I subunits, albeit with slightly lower efficiency in some cases (see Table 3). Expression of NuoL in *B. subtilis* $\Delta mrpA$ at pH 7.5 improved the growth substantially, but expression of NuoM or NuoN was not helpful at all to this strain (see Fig. 3A). At the same pH, wild type growth properties were regained by expression of NuoN in *B. subtilis* $\Delta mrpD$, whereas NuoL expression was less efficient (see Fig. 3B). NuoM was not complementing the growth very well in either *B. subtilis* $\Delta mrpA$ or $\Delta mrpD$ (Fig. 3A and B). Just as was previously seen in the *E. coli* proteins, the functional specificity was lost at a more acidic pH. At pH 6.5, all the *B. cereus* proteins could rescue both deletion strains to some extent. Likewise, at pH 8.4, none of the antiporter like Nuo proteins could complement the growth of the deletion strains

Table 3

Growth properties of *B. subtilis* deletion strains, expressing antiporter like proteins under different growth conditions.

	<i>B. subtilis</i> $\Delta mrpA$ at 80 mM Na ⁺			<i>B. subtilis</i> $\Delta mrpD$ at 60 mM Na ⁺		
	Max OD	g (min)	lag (h)	Max OD	g (min)	lag (h)
At pH 7.4						
<i>E. coli</i> complex I						
NuoL	1.74	73	0	1.58	115	2
NuoM	1.24	155	0	0.40	na	na
NuoN	0.43	na	na	1.77	76	2
<i>B. cereus</i> complex I						
NuoL	1.46	102	0	1.50	112	2
NuoM	0.57	na	na	0.56	na	na
NuoN	0.51	na	na	1.7	95	2
<i>E. coli</i> hydrogenase-3						
HycC	1.77	75	2	1.65	111	2
<i>E. coli</i> hydrogenase-4						
HyfB	1.38	108	1	1.53	178	1
HyfD	1.80	91	1	1.19	277	1
HyfF	1.73	83	1	1.32	195	1
<i>B. subtilis</i> Mrp						
MrpA	1.83	46	0	0.26	na	na
MrpD	0.27	na	na	1.88	41	2
At pH 6.5						
<i>E. coli</i> complex I						
NuoL	1.61	79	0	1.67	98	2
NuoM	1.52	91	0	1.62	87	1
NuoN	1.65	85	0	1.80	89	0
<i>B. cereus</i> complex I						
NuoL	1.78	115	0	1.75	102	2
NuoM	1.60	118	0	1.77	123	1
NuoN	1.76	100	0	1.81	96	0
<i>E. coli</i> hydrogenase-3						
HycC	1.71	75	0	1.80	88	0
<i>E. coli</i> hydrogenase-4						
HyfB	1.65	90	0	1.74	89	0
HyfD	1.73	96	0	1.80	98	0
HyfF	1.69	96	0	1.78	76	0
<i>B. subtilis</i> Mrp						
MrpA	1.74	71	0	1.02	110	2
MrpD	1.14	103	1	1.81	64	0
At pH 8.4						
<i>E. coli</i> complex I						
NuoL	0.12	na	na	0.14	na	na
NuoM	0.13	na	na	0.12	na	na
NuoN	0.10	na	na	0.14	na	na
<i>B. cereus</i> complex I						
NuoL	0.15	na	na	0.13	na	na
NuoM	0.12	na	na	0.09	na	na
NuoN	0.10	na	na	0.07	na	na
<i>E. coli</i> hydrogenase-3						
HycC	0.11	na	na	0.15	na	na
<i>E. coli</i> hydrogenase-4						
HyfB	0.11	na	na	0.11	na	na
HyfD	0.10	na	na	0.11	na	na
HyfF	0.10	na	na	0.11	na	na
<i>B. subtilis</i> Mrp						
MrpA	1.71	64	3	0.12	na	na
MrpD	0.10	na	na	1.67	56	6

(Table 3). The functional comparison of NuoL, NuoM and NuoN proteins from classical, 14-subunit complex I from *E. coli* and 11-subunit complex I from *B. cereus* (see Section 3.2, Fig. 3) corroborated the theoretical predictions (Section 3.2, Fig. 2) and supports the notion that 11-subunit complex I is a real, *bona fide* complex I enzyme. In analogy with the previous study [30], it can be concluded that the compact 11-subunit complex I from *B. cereus* most likely also contain a Na⁺ transporter subunit, NuoL, and two H⁺ transporter subunits, NuoM and NuoN. The recently discovered structural homologue NuoH [5] is not expected to conduct any ion transport, since any primary sequence similarity has since long been obliterated.

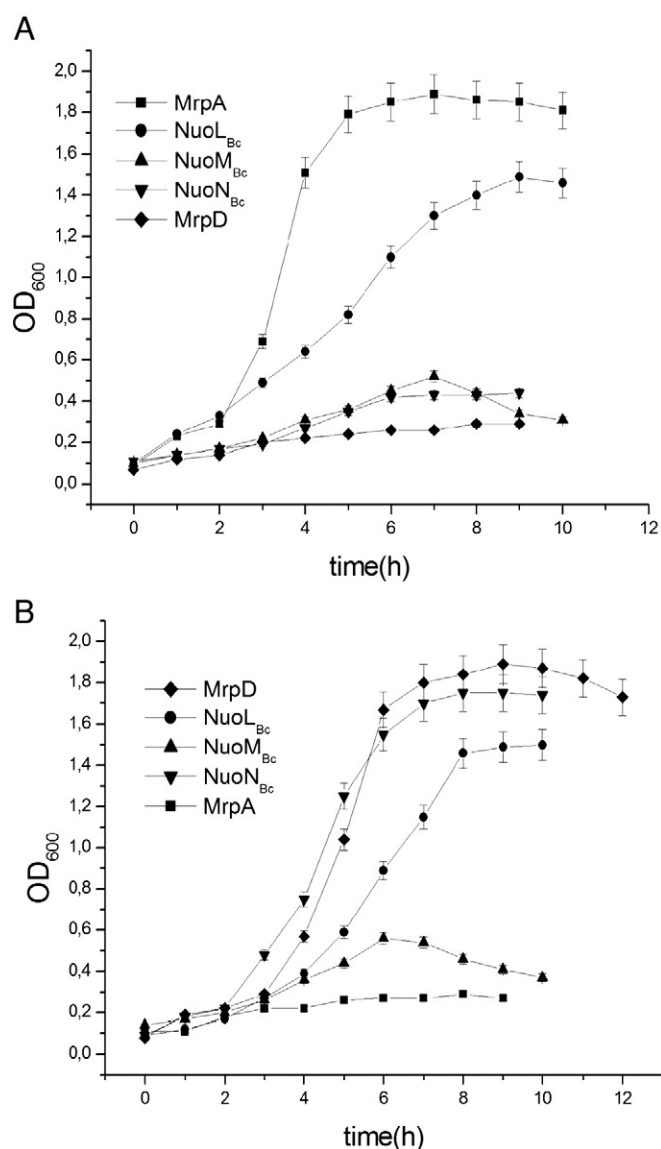


Fig. 3. Growth properties of *B. subtilis* deletion strains expressing *B. cereus* complex I proteins. Complementation of *B. subtilis* $\Delta mrpA$ by Nuol is shown in panel A and complementation of *B. subtilis* $\Delta mrpD$ by Nuon in panel B. The cells were grown aerobically in rich media (see Section 2.) at pH 7.4 and 80 mM NaCl (A) or 60 mM NaCl (B). In panel A, the growth of *B. subtilis* $\Delta mrpA$ /pVM15 expressing Nuol, is shown with filled circles, *B. subtilis* $\Delta mrpA$ /pVM16 expressing Nuom is shown with filled upward facing triangles and *B. subtilis* $\Delta mrpA$ /pVM17, expressing Nuon is shown with filled downward facing triangles. *B. subtilis* $\Delta mrpA$ /pVM11, and *B. subtilis* $\Delta mrpA$ /pVM6, expressing MrpA or MrpD respectively are shown with filled squares and filled diamonds for comparison. In Panel B, the growth of *B. subtilis* $\Delta mrpD$ /pVM15 expressing Nuol is shown with filled circles, *B. subtilis* $\Delta mrpD$ /pVM16 expressing Nuom is shown with filled upward facing triangles, *B. subtilis* $\Delta mrpD$ /pVM17, expressing Nuon is shown with filled downward facing triangles. *B. subtilis* $\Delta mrpD$ /pVM11, and *B. subtilis* $\Delta mrpD$ /pVM6, expressing MrpA and MrpD are shown in filled squares and diamonds respectively.

3.4. Complementation of *B. subtilis* $\Delta mrpA$ and $\Delta mrpD$ strains with ion transporter proteins from hydrogenase-3 and -4

To test the predictions also for a present day hydrogenase, the HycC subunit from *E. coli* formate:hydrogen lyase (a hydrogenase-3 enzyme, see Fig. 1 and Table 1), was cloned, cytochrome tagged and the construct was moved to the low copy number expression plasmid. The construct was transformed into *B. subtilis* $\Delta mrpA$ and *B. subtilis* $\Delta mrpD$ and the growth properties were investigated as before. Interestingly, HycC was able to complement both strains (Fig. 4), and to about the same extent at neutral and more acidic pH (Table 3). This suggests that the HycC

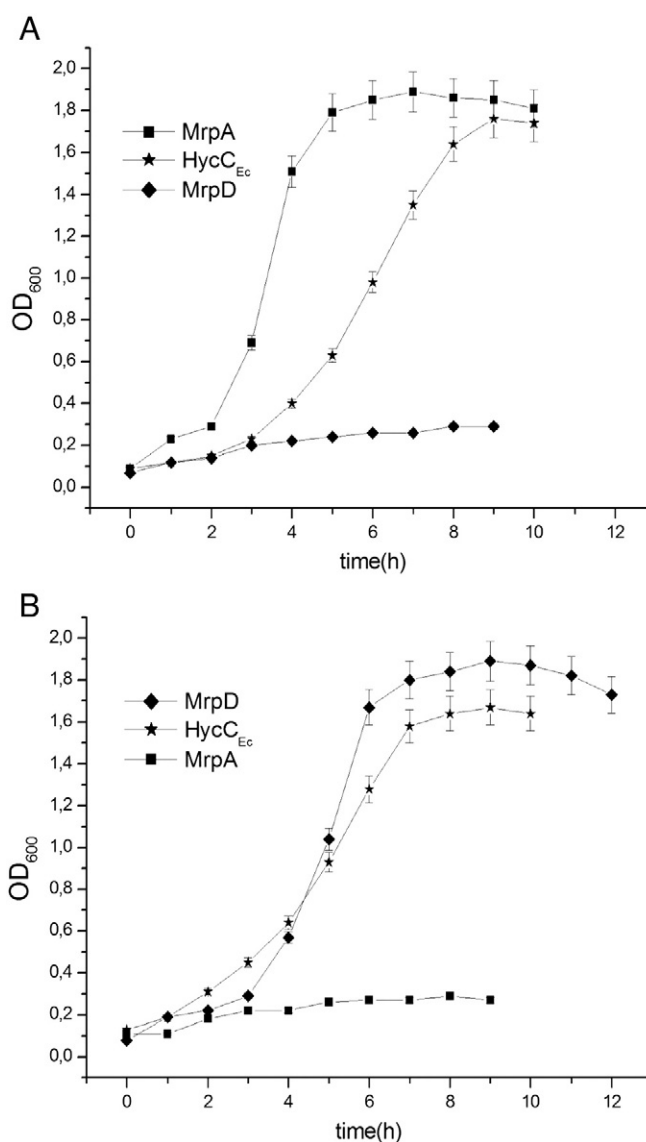


Fig. 4. Growth properties of *B. subtilis* deletion strains expressing the *E. coli* hydrogenase-3 subunit, HycC. Complementation of *B. subtilis* $\Delta mrpA$ by HycC is shown in panel A and complementation of *B. subtilis* $\Delta mrpD$ by HycC is shown in panel B. The cells were grown as in Fig. 3. In panel A, the growth of *B. subtilis* $\Delta mrpA$ /pVM20 expressing HycC, is shown with filled stars. The growth of *B. subtilis* $\Delta mrpA$ /pVM11, expressing MrpA (filled squares) and *B. subtilis* $\Delta mrpA$ /pVM6 expressing MrpD (filled diamonds) are shown for comparison. In panel B, *B. subtilis* $\Delta mrpD$ /pVM20 expressing HycC, is shown with filled stars. As in A, the growth of *B. subtilis* $\Delta mrpD$ /pVM11 and *B. subtilis* $\Delta mrpD$ /pVM6 is shown for comparison using the same symbols as before for the cells expressing harboring MrpA and MrpD respectively.

subunit exhibits as little ion specificity at pH 7.4 as the other ion transporters did at pH 6.5. As expected, the hydrogenase protein was not able to influence the growth of the deletion strains at pH 8.4, since these conditions seemingly require Mrp complex formation [30]. Very similar results were obtained for the three Hyf-proteins, showing no particular preference substituting for MrpA or MrpD (Table 3). Taken together, these results are in agreement with those predicted from sequence, i.e. that the hydrogenase ion transporter proteins seem to have much less functional specialization.

4. Discussion

The 11-subunit complex I, lacking the NuoE, F and G subunits, was previously shown to be the evolutionary progenitor of all complex I [14] since it was found scattered all over the phylogenetic “tree of life”, both

in archaea and in eubacteria. We have now demonstrated that the antiporter-like membrane spanning subunits NuoL, M and N of the compact complex I also behaves in the same way as the corresponding subunits from full size complex I, using a previously tested model system. This warrants the conclusion that 11-subunit complex I is a *bona fide* complex I enzyme, in spite that a different electron donor module is used.

We have previously concluded that the simplest explanation for the observed growth properties of the bacteria in the model system was that each protein comprised a single ion channel rather than a complete antiporter [30]. In this work we have demonstrated that NuoL could act as a Na⁺ channel whereas NuoN could not, further supporting the different functional properties of these homologous polypeptides. The assignment of MrpA/NuoL as a Na⁺ channel and MrpD/NuoN as a H⁺ channel was corroborated by the Na⁺-NMR experiments, suggesting that this functional specificity of the subunits is indeed related to their ion specificity.

Sequence comparisons [29] suggested that modern-day hydrogenases were lacking the signature features of MrpA or MrpD sequences, although all the proteins in the family are homologous. In the current work it was shown that the corresponding subunits from both hydrogenase-3 and hydrogenase-4 enzymes could substitute equally well either for MrpA or MrpD, corroborating the theoretical predictions (Fig. 2). In complex I we observe such loss of ion specificity at more acidic pH ([30], Table 3), but in hydrogenase subunits this feature seems to be always present. Either the functional specificity is not needed in a hydrogenase enzyme, or it is regulated differently than in complex I. In any case, the reasons for this difference remain to be elucidated. In addition, hydrogenases from other sources should be tested, to unravel if the observation is valid for all hydrogenases and if so, if the loss of ion specificity has happened multiple times over the course of evolution.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.bbabi.2013.09.012>.

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