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Homologous protein subunits from *Escherichia coli* NADH:quinone oxidoreductase can functionally replace MrpA and MrpD in *Bacillus subtilis*

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ABSTRACT

The complex I subunits NuoL, NuoM and NuoN are homologous to two proteins, MrpA and MrpD, from one particular class of Na^+/H^+ antiporters. In many bacteria MrpA and MrpD are encoded by an operon comprising 6–7 conserved genes. In complex I these protein subunits are prime candidates for harboring important parts of the proton pumping machinery. Deletion of either *mrpA* or *mrpD* from the *Bacillus subtilis* chromosome resulted in a Na^+ and pH sensitive growth phenotype. The deletion strains could be complemented *in trans* by their respective Mrp protein, but expression of MrpA in the *B. subtilis* Δ*mrpD* strain and *vice versa* did not improve growth at pH 7.4. This corroborates that the two proteins have unique specific functions. Under the same conditions NuoL could rescue *B. subtilis* Δ*mrpA*, but improved the growth of *B. subtilis* Δ*mrpD* only slightly. NuoN could restore the wild type properties of *B. subtilis* Δ*mrpD*, but had no effect on the Δ*mrpA* strain. Expression of NuoM did not result in any growth improvement under these conditions. This reveals that the complex I subunits NuoL, NuoM and NuoN also demonstrate functional specializations. The simplest explanation that accounts for all previous and current observations is that the five homologous proteins are single ion transporters. Presumably, MrpA transports Na^+ whereas MrpD transports H^+ in opposite directions, resulting in antiporter activity. This hypothesis has implications for the complex I functional mechanism, suggesting that one Na^+ channel, NuoL, and two H^+ channels, NuoM and NuoN, are present.

1. Introduction

NADH:quinone oxidoreductase (complex I) consists of 14 dissimilar protein subunits, most of them homologous to proteins found elsewhere, reflecting the evolution of the large enzyme complex from smaller functional building blocks (see Fig. 1). 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. That the three complex I subunits NuoL/NQO12/ND5, NuoM/NQO13/ND4 and NuoN/NQO14/ND2 were similar to each other was noticed by Fearnley and Walker already in 1992 [1], whereas the homology to a particular type of antiporter protein found in an alkalophile *Bacillus* sp. was discovered by Hamamoto et al. [2]. This type of antiporter, denoted Mrp/Pha/Sha/Mnh, has since been found also in many mesophile bacteria [3–7], and is typically encoded by a conserved operon structure consisting of six or seven genes, *mrpABCDEFG*. *MrpA* and *mrpB* can be separate genes or be fused into one longer *mrpA* [8]. These antiporters constitute their own family (cation proton antiporter-3, CPA-3) and also hold a unique transporter classification (TC.2.A.63) [9]. Of the Mrp proteins, both MrpA and MrpD are homologous to NuoL, NuoM and NuoN. Initially the evolution of this part of complex I was envisioned as triplication of one acquired gene [10]. A more detailed sequence comparison revealed that although all five proteins are homologous, they each have some unique conserved sequence motifs as well. The NuoL group also has features that more closely resembles MrpA whereas NuoM and NuoN are more similar to MrpD [11]. In addition, the NuoK/NQO11/ND4L was found to be homologous to MrpC, indicating that a whole MrpABCD module rather than a single protein was recruited to complex I [12].

To understand the energy coupling mechanism of complex I, it is essential to learn more about the proton translocation process. As antiporter homologues, the NuoL, NuoM and NuoN are prime candidates for harboring the means for transmembrane proton translocation. Furthermore, there have been a number of studies over the years that imply that Na^+ or K^+ may somehow be involved in the functional mechanism, both in complex I [13–16] and in the membrane bound NiFe-hydrogenase [17]. In a study by Nakamaru-Ogiso et al. [18], amilorides, that are believed to inhibit Na^+/H^+ antiporters by binding to the Na^+ site, inhibited the energy-coupled activities of bovine heart complex I. It has however not been known how much of the primordial functions remain in the antiporter-like complex I subunits and if they retain the capability to translocate Na^+ or K^+ as well as H^+, or if they even preserve a true antiporter function.

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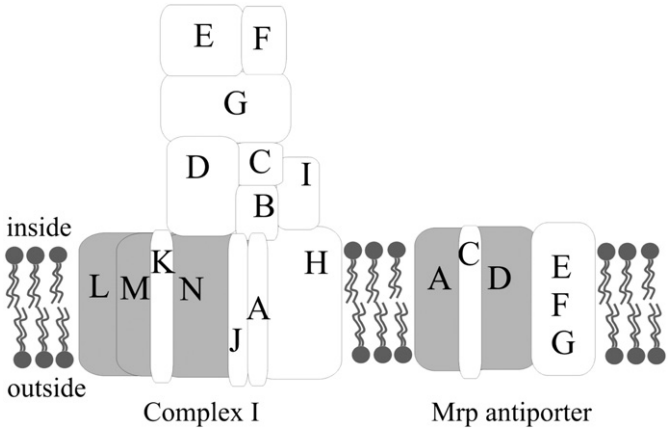

Fig. 1. Schematic representation of the 14 protein subunits of complex I (left) and the 6 protein subunits of the Mrp antiporter complex (right). The "antiporter-module" of complex I comprises the large membrane spanning NuoL, NuoM and NuoN subunits, that are homologous to the MrpA and MrpD subunits of the *bona fide* antiporter, and the smaller NuoK that is homologous to MrpC [12]. The location of the membrane spanning complex I subunits is tentative. The NuoL and NuoM subunits are most likely the most peripheral subunits, since they could be split off from the rest of the enzyme [56]. The Mrp antiporter complex contains 6 or 7 proteins, since MrpB can either be a separate protein or be fused with MrpA. The MrpABCD can form a subcomplex [26] but changes in either of the two Mrp modules MrpA-D and MrpE-G influence the other. MrpE was shown to be particularly important for complex formation and overall stability [34].

To complicate things further, although tremendous progress has occurred in the recent year, structural information about these proteins is still very limited. A recently solved structure of the membrane-domain of complex I from *Escherichia coli* revealed NuoL, NuoM and NuoN as structurally nearly identical entities, and showed the presence of a pair of interesting half helix features in these structures that probably are central to the ion translocation mechanism [19]. Unfortunately, the relatively low resolution and absence of electron densities in the extra-membranous loop regions in both this and a subsequent structure of eukaryote complex I from *Yarrowia lipolytica* [20] makes it impossible to identify the location of these half-helix elements in the primary sequence and relate the putative function to the position of mutations known to affect function [21–23]. There is no crystal structure at all available for the Mrp antiporter complex. The Mrp proteins were suggested to form a hetero-oligomeric complex, since antiporter activity seemed to depend on the presence of all 6 or 7 proteins [7,24]. Subsequently, the polypeptides encoded by the *mrp* operon were tagged with c-terminal extensions that allowed reliable detection of each protein subunit. Thereby it was possible to differentiate between stability effects and antiporter activity effects of deletions or point mutations, establishing the requirement for Mrp protein complex formation for antiporter activity [25,26]. Functionally, both MrpA and MrpD were shown to have a role in Na^+ resistance and Na^+ dependent pH homeostasis early on [24,27,28]. Whole cell experiments and membrane vesicle analyses have shown that the Mrp antiporter complex is a secondary antiporter, that is energized by the membrane potential [8,29–31]. A number of site directed mutant studies of conserved residues have identified several acidic residues essential for function, that putatively could form ion translocation sites [32,33] and fully conserved lysines have been implicated both in MrpA, MrpD [34] and NuoM [21].

In this work we are using *B. subtilis* strains where MrpA or MrpD has been genetically removed, to investigate the ion translocation abilities of the individual NuoL, NuoM and NuoN complex I subunits from *E. coli*. Under some conditions the complex I proteins could rescue the salt and pH sensitive deletion strains, demonstrating that complex formation is not universally obligatory for function of the Mrp antiporter. It could also be concluded that the complex I proteins have retained important primordial elements of their functional mechanism. At pH 7.4, MrpA could be functionally replaced by NuoL whereas MrpD was instead complemented by NuoN, corroborating the predictions previously obtained by primary sequence analyses [11].

2. Materials and methods

2.1. Molecular biology

Bacterial strains, plasmids and primers used are listed in Table 1. *E. coli* cells were grown aerobically at 37 °C and 200 rpm in LB medium [35]. 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; [36]) and were kept on Tryptose Blood Agar Base plates (TBAB, Difco). For *B. subtilis* growth studies, see Section 2.4. Antibiotic was added in the following concentrations when appropriate: 100 μg/ml ampicillin, 12.5 μg/ml chloramphenicol and 10 μg/ml kanamycin of for *E. coli*, 5 μg/ml chloramphenicol and 2 μg/ml phleomycin (Duchefa) for *B. subtilis*. Electrocompetent *E. coli* were prepared as described in [35], and the cells were subsequently transformed by electroporation using a BioRad *E. coli* Pulser™ transformation apparatus. Chemical transformation of *E. coli* with $CaCl_2$ was done as described by Mandel and Higa [37]. *B. subtilis* strains were grown to competence as described by Arwert and Venema [38], using a fresh plate with confluently growing bacteria for inoculation of 50 ml resuspension media.

Standard recombinant DNA procedures were done as described in [35]. Primers were synthesized by MWG Biotech AB, Germany, TAG Copenhagen, Denmark, Sigma-Genosys, and Fermentas life sciences. The sequences of the primers used are listed in Table 1. PCR reactions were done in a PTC-200 peltier thermal cycler. Restriction enzymes and dNTPs were from Invitrogen, New England BioLabs (NEBL) or MBI Fermentas. PCR products were purified from agarose gel with Jetsorb gel extraction kit (Genomed) and restriction enzyme digested vectors and DNA fragments were purified by DNA Clean Up (Genomed) or Jetsorb. DNA ligation was performed in 10 μl reactions containing 50–150 ng DNA at 16 °C over night or at 4 °C for 24–30 h, using T_4 DNA ligase from Fermentas and NEBL. Small-scale preparations of plasmids were done with as mini plasmid preparation kits by Fermentas, while large-scale plasmid preparations were done using the Wizard Plus Midipreps (Promega). Chromosomal DNA from *B. subtilis* strains was prepared as described by Marmur [39] and for chromosomal DNA from *E. coli* the method of Wilson was used [40]. DNA sequencing reactions were done using Big Dye™ (Applied Biosystems) and the subsequent sequence analysis was performed at the Biomolecular Resource Facility, Lund University.

2.2. Construction of B. subtilis deletion strains

Deletion of *mrpA* and *mrpD* from the *B. subtilis* chromosome by homologous recombination was done essentially as described in [41]. Two regions of DNA flanking the gene targeted for deletion were cloned upstream and downstream of the *ble* gene in pBle1 [42]. pBle1 is a pUC18 derivative that is not able to replicate in *B. subtilis*. Thus, a phleomycin resistant phenotype can only result when the *ble* gene has been inserted in the *B. subtilis* chromosome by homologous recombination. The flanking regions were chosen such that the neighboring genes in the gene cluster would not be affected by the insertion of the *ble* gene. The respective flanking regions were amplified from *B. subtilis* 3G18 chromosomal DNA using Red Taq polymerase (Sigma). The 50 μl PCR reactions included 600 ng template DNA and 3.5 mM $MgCl_2$. Common for these PCR programs were melting at 94 °C 3 min, 25 cycles of 94 °C 30 s, varying annealing temperature, 45 s, elongation 72 °C 40 s and a finish including 94 °C 30 s, 72 °C 5 min and 8 °C forever. The following annealing temperatures and primers (see also Table 1) were used: 46 °C, mrpAdelPstIUp, mrpAdelUpSalI (671 base pairs, bp); 54 °C, mrpAdelKpnIDo, mrpAdelDoEcoRI (692 bp); 51 °C, mrpDdelPstIUp, mrpDdelUpSalI (677 bp); 48 °C, mrpDdelKpnIDo, mrpDdelDoEcoRI

Table 1
Bacterial strains, plasmids and primers used in this work.

Bacterial strain	Genotype	Reference/source
B. subtilis 168A	Wild type, (type train), *trpC2*	*Bacillus* Genetic Stock Center
B. subtilis Δ*mrpA*	Δ*mrpA* ble^r	This work
B. subtilis Δ*mrpD*	Δ*mrpD* ble^r	This work
E. coli JM109	*endA1 glnV44 thi-1 relA1 gyrA96 recA1 mcrB$^+$ Δ(lac-proAB) e14-[F′ traD36 pro AB$^+$ lacI^q lacZΔM15] hsdR17(r_k^- m_k^+)*	[44]
E. coli XL1-Blue	*recA1, endA1, gyrA96, thi, hsdR17, supE44, relA1 (lac)*	Promega
E. coli GM3819 (Dam$^-$)	dam-16::Kan thr-1 leuB6 thi-1 argE3 hisG4 proA2 lacY1 galK2 mtl-1 xyl-5 ara-14 rpsL31 tsx-33 supE44 rfbD1 kdgK51	[42]
Plasmids	**Relevant properties**	
pCW6	Cm^r	Claes von Wachenfeldt
pΔmrpA	*lacZ′*, Bla^r, Ble^r, Amp^r	This work
pΔmrpD	*lacZ′*, Bla^r, Ble^r, Amp^r	This work
pBle1	*lacZ′*, Bla^r, Ble^r, Amp^r	[41]
pLC1	*nuoLcyt*, Amp^r	[43]
pMC1	*nuoMcyt*, Amp^r	[43]
pNC1	*nuoNcyt*, Amp^r	[43]
pVM11	*mrpAcyt*, Cm^r	[43]
pVM6	*mrpDcyt*, Cm^r	[43]
pVM8	*nuoLcyt*, Cm^r	This work
pVM9	*nuoMcyt*, Cm^r	This work
pVM10	*nuoNcyt*, Cm^r	This work
pVM7	*mrpABCDcyt*, Cm^r	This work
pVM26	*nuoKLMNcyt*, Cm^r	This work
Primers	**Restriction enzyme site**	**Primer sequence**
nuoL_Rev_pCW6	*Sal*I	5′ CCGCTACTGTCGACAATCGTTTA 3′
nuoLM_For_pCW6	*Xba*I	5′ CAGCTCTAGACATGATTACGCC 3′
nuoMN_Rev_pCW6	*Pst*I	5′ CCGCTACTGTCTGCAGTCGTTTA3′
nuoN_For_pCW6	*Xba*I	5′ GTGGTTTGTCTAGACCGTTACTAC 3′
mrpABCD_Up	*Xba*I	5′ CGCATCTAGATTGCAGCTCTTAC 3′
mrpABCD_Dwn	–	5′ CGTAATAAGAGCCTGCAGGCCAAAAG 3′
nuoKLMN_For	*Xba*I	5′ CGTAAAGTCTAGAGCGCGAAAAG 3′
nuoKLMN_Rev	–	5′ GTAGTAGAGGCCGATTGCCGAAC 3′
mrpAdelUpUp		5′ GTATTAGACTGCAGTCCGTCACTC 3′
mrpAdelUpDo		5′ GTAAAGAGGTCGACGAGGGAC 3′
mrpAdelDoUp		5′ GATCGGTACCATTGGTTATTTGTCG 3′
mrpAdelDoDo		5′ CAAATCCCCTGAATTCAACGAGAATG 3′
mrpDdelUpUp		5′ GAATCTGCAGCACGGCGGC 3′
mrpDdelUpDo		5′ CGCATATAAGTCGACTTTTGTCAGC 3′
mrpDdelDoUp		5′ GGATTGGGTACCGAATGGG 3′
mrpDdelDoDo		5′ GACACGAATTCCATACAAAAAGG 3′

(646 bp). The pBle1 vector and the respective downstream fragments were cut with *Kpn*I and *Eco*RI. Vector and fragments were ligated, resulting in constructs with the respective fragment inserted upstream the phleomycin resistance gene in pBle1. The new constructs and the respective upstream fragments were then digested with *Pst*I and *Sal*I and ligated, resulting in constructs with the second fragments inserted downstream the phleomycin resistance gene. The final plasmid constructs were named pΔmrpA and pΔmrpD, respectively. Presence of the correct inserts was verified by colony PCR as described in [11] and by restriction enzyme analysis of plasmids. Competent *B. subtilis* 168A was transformed to phleomycin resistance with pΔmrpA and pΔmrpD, respectively. Deletion of *mrpA* or *mrpD* was verified by PCR amplification of the modified DNA region, using 25 μl PCR reactions containing ~70 ng chromosomal DNA from the respective strain as a template: *B. subtilis* Δ*mrpA* chromosomal DNA was analyzed using the primers mrpAdelPstIUp and mrpAdelDoEcoRI, annealing temperature 46 °C, 2 min 30 s amplification and *B. subtilis* Δ*mrpD* was analyzed with the primers mrpDdelPstIUp and mrpDdelDoEcoRI, annealing temperature 41 °C, 2 min 30 s amplification, in both cases using Red Taq Polymerase.

2.3. Construction of the pVM8, pVM9, pVM10, pVM7 and pVM26 expression plasmids

The pLC1, pMC1 and pNC1 plasmid DNAs were used as template for amplification of *nuoLcytc*, *nuoMcytc* and *nuoNcytc* respectively, using the primers shown in Table 1. In the downstream primers, a unique *Pst*I site was introduced, except in *nuoLcytc* that contain an internal *Pst*I site, and therefore *Sal*I was used instead. In the upstream primers a *Xba*I site was created in all the constructs. The PCR conditions were 3 min initial denaturation at 95 °C followed by 26 cycles of 45 s at 95 °C, 45 s annealing at 45 °C for *nuoLcytc* and *nuoMcytc* and 46 °C for *nuoNcytc* and elongation at 72 °C for 3, 2 and 2 min respectively for the three constructs. *Dream Taq* DNA polymerase was used. The shuttle expression vector pCW6 was purified from *E. coli* GM3819 [43] to obtain unmethylated plasmid DNA that can be digested with *Xba*I. The *nuoMcytc* and *nuoNcytc* PCR product were digested with *Pst*I and *Xba*I and ligated into previously *Pst*I and *Xba*I digested pCW6. For *nuoLcytc* *Sal*I was used instead. The ligate was transformed into *E. coli* JM109, and plasmids prepared from a couple of transformants were subjected to DNA sequencing. The resulting correct constructs were named pVM8, pVM9 and pVM10.

To build plasmid pVM7 the *mrpABCD* region was amplified from *B. subtilis* chromosomal DNA by using the primers shown in Table 1. The upstream primers contains were created with a *Xba*I site whereas the downstream primer did not contain any restriction site sequence. The PCR reaction was run using *Dream Taq* polymerase under the following conditions; 3 min initial denaturation at 95 °C followed by 26 cycles comprising 45 s denaturation at 95 °C, 45 s annealing at 54 °C, initial elongation at 72 °C for 5 min, followed by a prolonged elongation time of 10 min. The *mrpABCD* PCR product was digested with *Xba*I and *Bsp*1407, and was ligated into plasmid pVM6 ([44]; Table 1) that had been opened with *Xba*I and the unique restriction

site *Bsp*1407 that cuts internally in *mrpD*. Plasmid pVM26 was constructed in essentially the same way, except that the *nuoKLMN* region was amplified from *E. coli* chromosomal DNA and the primers listed in Table 1. The PCR conditions were 3 min and 30 s initial denaturation at 95 °C followed by 26 cycles of 45 s denaturation at 95 °C, 45 s annealing at 47 °C, initial elongation at 72 °C for 5 min followed by a prolonged elongation of 10 min as before. The *nuoKLMN* PCR product was digested with *Xba*I and the restriction enzyme *Blp*I, that cuts within the *nuoN* gene. The fragment could therefore be ligated into the previously constructed pVM10 that had been opened with *Xba*I and *Blp*I. The constructs were transformed into *E. coli* JM109 [45] and confirmed by sequencing as before.

2.4. B. subtilis growth studies

The *B. subtilis* strains were grown in 35 ml batches at 37 °C with shaking at 200 rpm in 250 ml baffled E-flasks with an attached sidearm consisting of 16 mm glass tube. This allowed convenient measurement of the optical density (OD) of the culture without opening the flasks or changing the growth medium volume throughout the experiment. The OD was measured every hour at 600 nm using a biowave cell density meter C08000, which has an error rate of $<\pm 0.05$ A at 1 A. The growth medium contained 8 g/l Nutrient broth (NB, Difco, Becton Dickinson Co.), and 5 ml/l of a metal mixture containing 0.14 M $CaCl_2$, 0.01 M $MnCl_2$ and 0.2 M $MgCl_2$. Chloramphenicol (5 μg/l) was present to retain the plasmid vectors. Since the expressed proteins harbor a cytochrome *c* domain, 1 μM $FeCl_3$ was also included in the growth medium. To maintain a constant pH 100 mM Tris–HCl was present in the growth media at pH 7.4 and pH 8.4 whereas 200 mM Tris–HCl was used during growth at pH 6.4. According to the manufacturer, the NB contains 6.84 mM Na^+ and 4.46 mM K^+ when used as recommended (8 g/l). The NaCl concentrations indicated in each experiment always refers to the amount of NaCl added to the growth medium. The *B. subtilis* strains to be studied were taken from glycerol stock cultures at −80 °C, and streaked on solid media containing the NB growth medium described above at pH 7.4 with no added NaCl and 1.5% agar. The plates were incubated at 37 °C for 8 h, and the cells were then used immediately to inoculate the liquid cultures. Isopropyl-Thio-β-D-Galactoside (IPTG, 1 mM) for induction of protein expression from the P_{spac} promotor was present from the time of inoculation. After the growth experiment was completed, the pH of the media was re-measured and a sample of the culture was re-streaked to ensure that contamination was absent. Most of the growth studies were repeated several times, but all experiments were repeated in at least two independent experiments.

Growth curves for each strain were obtained by plotting the measured OD values against time. The generation time *g* is the time it takes for a bacterium 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 the corresponding time points during logarithmic growth in minutes, and *k* is a growth constant.

$$k = ln(OD2) - ln(OD1) / t2 - t1 \quad (1)$$

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

2.5. Quantification of the expressed proteins

Wild type *B. subtilis* were grown in single 400 ml batches in NSMP at 37 °C and 200 rpm in 2 l baffled E-flasks. Protein expression from plasmids was induced by adding 1 mM IPTG when the culture had reached an OD_{600} of 0.4. The bacteria were harvested in early stationary phase. The deletion strains were grown in the same buffered media as in Section 2.4 at pH 7.4 and 100 mM NaCl. IPTG was present from the start. Several identical 400 ml batches were grown simultaneously in 2 l baffled E-flasks, after which one batch was harvested every hour, beginning in the mid-log-phase. Upon harvest, the cells were washed in 50 mM KPO_4 buffer pH.8.0, and the cell pellets were stored at −20 °C until needed. Membranes were prepared as described [46], frozen in liquid N_2 and were stored at −80 °C. The protein concentration of each membrane preparation was determined using BCA method (Pierce), including 4% SDS in the reactions and using bovine serum albumin as standard. SDS-PAGE was done according to Neville [47]. The membranes were thawed, and solubilized in 4% SDS at room temperature for 30 min, incubated with loading buffer at 37 °C for 45 min, and were subsequently loaded on the gel. The electrophoresis was run at 80 V for 3 h. Western blot, using anti-cytochrome c_{550} antiserum as primary antibody, was done as described previously [44]. The polyacrylamide gels were stained with Coomassie after blotting to confirm the efficiency of protein transfer. The cytochrome c_{550} concentration in the membranes, that was used as an internal standard when estimating the fusion protein content, was determined spectroscopically using the extinction coefficient for cytochrome c_{550}, $\epsilon_{550-535} = 24\ mM^{-1}\ cm^{-1}$ [48].

3. Results

3.1. Properties of the B. subtilis ΔmrpA and ΔmrpD strains comprising the model system

B. subtilis mrpA and *mrpD* deletion strains were created by homologous recombination between the chromosome and plasmid DNA. The deletion plasmids, pΔmrpA and pΔmrpD (Table 1) contain the flanking regions of each of the targeted genes with the phleomycin resistance gene in between. The pΔmrpA and pΔmrpD plasmids are unable to replicate in *B. subtilis*. Thus, to exhibit a phleomycin resistant phenotype, the *ble* gene has to be incorporated into the *B. subtilis* chromosome by double cross-over between plasmid and chromosomal DNA, subsequently removing the target gene. The phleomycin resistance gene was inserted in the reverse direction compared to the *mrp* genes on the chromosome, to ensure a normal transcription of genes downstream of the deleted segment. The correct insertion of *ble* was confirmed by PCR.

The strain phenotypes were investigated in a rich medium buffered by 100 or 200 mM TRIS (see Section 2.4). As expected from earlier data using similar deletion strains [49], the constructed *B. subtilis* Δ*mrpA* and *B. subtilis* Δ*mrpD* deletion strains showed a salt and pH-sensitive growth phenotype (Fig. 2) and the salt sensitivity became more severe at more alkaline pH. At pH 8.4 neither strain could tolerate even 25 mM NaCl in the growth medium. The Δ*mrpA* strain was able to grow somewhat at 25 mM NaCl at slightly acidic or neutral pH but at 60 mM NaCl the growth was very poor (Fig. 2A). The Δ*mrpD* strain behaved similarly, but ceased to grow already at 40 mM NaCl (Fig. 2B). Thus, we conclude that the Δ*mrpD* strain is overall somewhat more Na^+ sensitive than the Δ*mrpA* strain. Based on these results, the standard growth conditions for testing complementation of the deletion strain phenotypes were set at 80 mM NaCl for the *B. subtilis* Δ*mrpA* strain and 60 mM NaCl for the *B. subtilis* Δ*mrpD* strain. It should be mentioned that the nutrient broth in itself contains about 7 mM Na^+ (see Section 2.4). This amount of salt is present in addition to the 80 and 60 mM added NaCl in all our experiments.

3.2. Expression of MrpA and MrpD in trans in the B. subtilis deletion strains

Subsequently the MrpA and MrpD proteins were expressed from a plasmid in the *B. subtilis* Δ*mrpA* (Fig. 3A) and *B. subtilis* Δ*mrpD* (Fig. 3B) deletion strains. To create physiologically relevant conditions, we used an expression system with a low copy number plasmid containing the IPTG inducible P_{spac} promotor ([50]; Table 1). To be able to monitor and compare the amounts of protein produced we

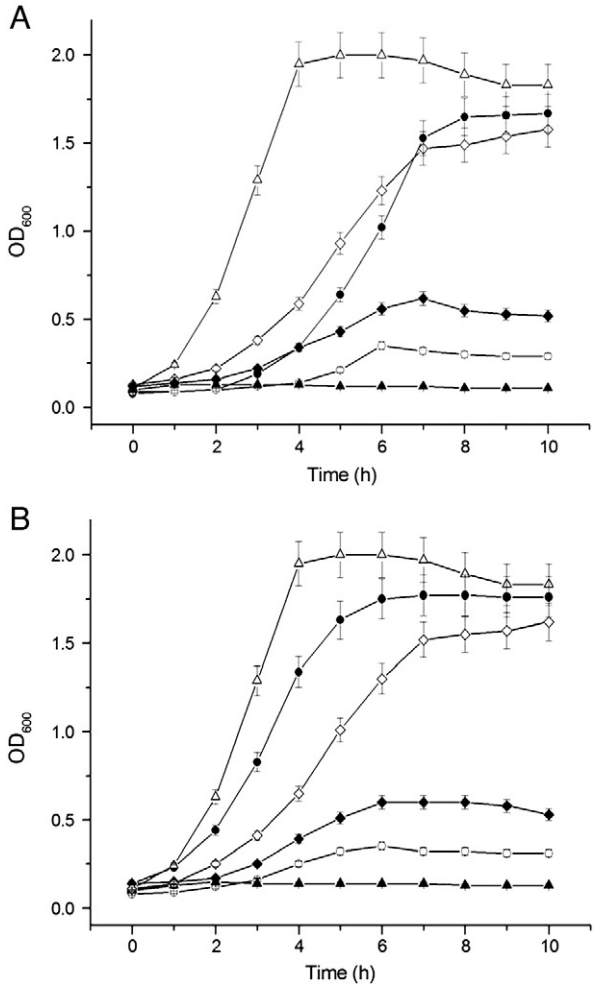

Fig. 2. Growth properties of the antiporter deletion strains *B. subtilis* Δ*mrpA* (panel A) and *B. subtilis* Δ*mrpD* (panel B) at different pH and salt concentrations. The cells are grown aerobically in rich media (see Section 2.4) and the pH and NaCl concentrations indicated below. *B. subtilis* 168 (wild type, open triangles) grown at pH 7.4 and 60 mM NaCl is shown for comparison in both panels A and B. In panel A, *B. subtilis* Δ*mrpA* grown in 25 mM NaCl at pH 6.5 is represented by filled circles, at pH 7.4 with open diamonds and at pH 8.4 as filled triangles. The same strain grown in 60 mM NaCl and pH 6.5 is shown as open circles and at pH 7.4 illustrated with filled diamonds. In panel B, the growth properties of *B. subtilis* Δ*mrpD* are shown. At 25 mM NaCl and pH 6.5 the growth curve is indicated by filled circles, at pH 7.4 it is shown with open diamonds and at pH 8.4 as filled triangles. The same strain grown in 40 mM NaCl at pH 6.5 is shown with open circles and at pH 7.4 with filled diamonds.

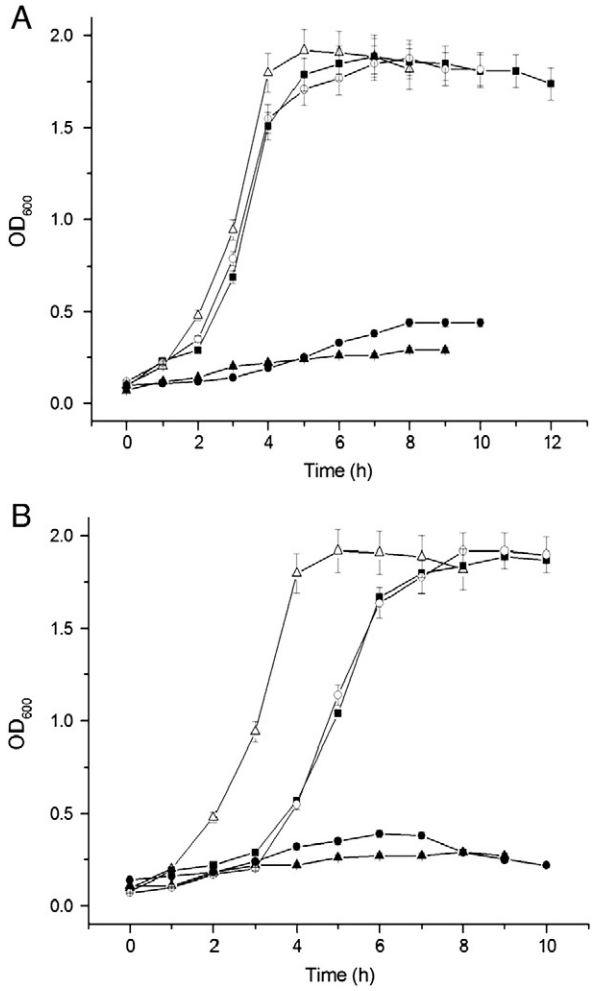

Fig. 3. Expression of the cytochrome *c*-tagged MrpA and MrpD proteins in the *B. subtilis* Δ*mrpA* (panel A) and *B. subtilis* Δ*mrpD* (panel B) deletion strains at pH 7.4. The cells were grown as in Fig. 2, with protein expression induced from the point of inoculation. In panel A, *B. subtilis* Δ*mrpA*/pVM11 that is expressing MrpA is shown when grown at 100 mM NaCl (filled squares) and at 80 mM NaCl (open circles). *B. subtilis* Δ*mrpA*/pVM6, that is expressing MrpD, is grown at 80 mM NaCl and represented by filled triangles. *B. subtilis* Δ*mrpA*/pCW6, the empty expression vector, grown at 80 mM NaCl is shown with filled circles. In panel B, *B. subtilis* Δ*mrpD*/pVM6 expressing MrpD, grown at 100 mM NaCl (filled squares) and 60 mM NaCl (open circles) is compared with *B. subtilis* Δ*mrpD*/pVM11 expressing MrpA and grown at 60 mM NaCl (filled triangles) and *B. subtilis* Δ*mrpD*/pCW6 harboring the empty expression, also at 60 mM NaCl (filled circles). In both panels, *B. subtilis* 168 (wild type, open triangles), grown at 100 mM NaCl is shown for comparison.

used the cytochrome *c* fusion domain previously devised by us [44] to tag all plasmid-expressed proteins. The amount of MrpA and MrpD fusion protein could then be estimated from Western blots. At the end of the growth studies (10–12 h) no MrpA or MrpD protein could typically be detected (not shown). When the cells were instead harvested in early stationary growth phase, low and comparable amounts of protein could be detected by Western blot. The endogenous cytochrome c_{550} was used as an internal standard, to estimate the content of the fusion proteins. We could never detect any higher amounts than 0.2 μg fusion protein/mg total membrane protein, even when the proteins were expressed in wild type *B. subtilis*, as exemplified in Fig. 4A. The fusion protein expression in the deletion strains was subsequently followed systematically from mid-log phase to stationary phase, by growing several larger batches of cells under the same conditions as were used in the growth studies. From mid-log phase, one batch of cells were harvested every hour, membranes were prepared, and subjected to Western blot as before. Only low amounts of protein could be detected, as exemplified in Fig. 4B, showing the amount of MrpA expressed in *B. subtilis* Δ*mrpA*. In summary, it was not meaningful to attempt to correlate the amount of expressed proteins with activity, since the protein amounts were typically around or below the detection level. More importantly, it could be concluded that, at no stage of the growth experiments, were there very high, unphysiologic amounts of expressed protein present in the cells.

At pH 7.4 the MrpA protein expressed *in trans* could fully restore the growth properties of *B. subtilis* Δ*mrpA* (Fig. 3A) but had no effect at all on the growth of *B. subtilis* Δ*mrpD* (Fig. 3B). Likewise, MrpD expressed *in trans* could rescue *B. subtilis* Δ*mrpD*, albeit after a ca. 2-h lag phase compared to wild type cells (Fig. 3B), but in *B. subtilis* Δ*mrpA* expression of MrpD was comparable to cells containing only the empty vector (Fig. 3A). The distinct primary sequence of MrpA and MrpD, and the presence of both *mrpA* and *mrpD* in the canonical conserved gene cluster, predicts that the two proteins should have somewhat different function. The slightly different salt sensitivity of the two deletion strains corroborates this notion (see Section 3.1, Fig. 2). It can also be concluded that a putative mild overexpression

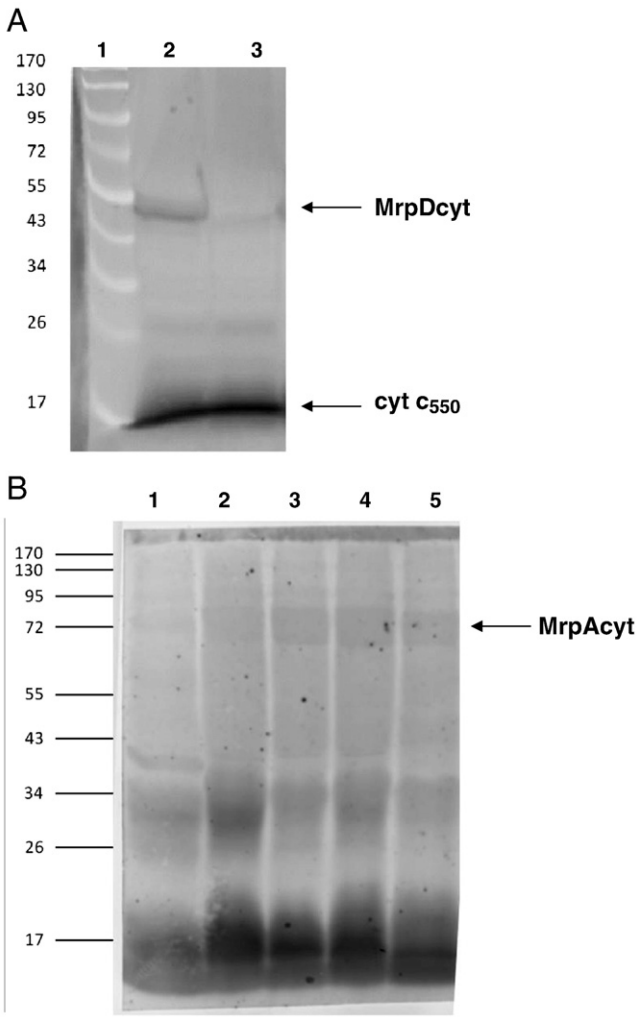

Fig. 4. A. Western blot of *B. subtilis* membranes. On the gel was loaded a protein size ladder (lane 1), membranes from cell expressing MrpDcyt (lane 2) and wild type membranes (cells containing only the expression vector, pCW6, lane 3). Total membrane protein (60 μg) was loaded in each lane. The proteins were separated in a 12% polyacrylamide gel. The cytochrome c_{550} content in the membranes, that were used as an internal standard, was determined spectroscopically [48] using the extinction coefficient for cytochrome c_{550}, $\varepsilon_{550-535} = 24\ mM^{-1}\ cm^{-1}$. B. Western blot of membranes from *B. subtilis* Δ*mrpA* expressing MrpAcyt from plasmid pVM11. The cells were grown as described in Section 2.5 at 100 mM NaCl. The membranes in lane 1 are from cells in mid-log phase, harvested after 3 h of growth, lane 2: 4 h, lane 3: 5 h, lane 4: 6 h and lane 5: 7 h. Total membrane protein (100 μg) was loaded in each lane. The cytochrome c_{550} content was determined as in A. The proteins were separated in a 10% polyacrylamide gel.

and/or absence of proper Mrp complex formation did not enable MrpA to replace MrpD or *vice versa* (Fig. 3) and thus both proteins maintain their individual functions in the *B. subtilis* model system. During these experiments it was noticed that using a fresh preculture was very important for obtaining consistent, reproducible growth results. As also mentioned in [49], older bacteria can develop an increased Na^+ tolerance.

3.3. Expression of the complex I subunits NuoL NuoM and NuoN in the *B. subtilis* deletion strains

The model system was subsequently implemented to investigate the antiporter-like proteins from *E. coli* complex I. The genes encoding NuoL, NuoM and NuoN where cytochrome *c* tagged and the fusion constructs were cloned into the same expression vector as was used to express MrpA and MrpD *in trans* in the deletion strains. Expression of the complex I subunits in the deletion strains could, in some combinations, restore the wild type growth properties (Table 2). *B. subtilis* 168A, hereafter denoted wild type, typically show a max OD of around or slightly more than 2 (the upper limit of our OD measuring devise) irrespectively of the growth medium pHs that are used here. The calculated mean generation time of these wild type bacteria were 46 min at pH 7.4, 49 min at pH 6.5, and 41 min at pH 8.4. Expression of any of the proteins MrpA, MrpD, NuoL, NuoM, NuoN in wild type *B. subtilis* had no effect on the growth properties at any pH or salt concentration used in this study.

The most efficient complementation of the deletion strains occurred when NuoL was expressed in *B. subtilis* Δ*mrpA* and when NuoN was expressed in *B. subtilis* Δ*mrpD* (Fig. 5A and B). Interestingly, this corroborates the similar function of MrpA and NuoL and the similar function of MrpD and NuoN that was predicted from sequence comparisons [11]. NuoM, on the other hand, was also predicted to resemble MrpD, but expression of NuoM was not helpful to *B. subtilis* Δ*mrpD* (Table 2). Some slight growth improvement was seen when expressing NuoM in *B. subtilis* Δ*mrpA*, but compared to expression of NuoL, the effect was quite modest. This demonstrated that the antiporter-like complex I proteins, at least NuoL and NuoN, still retain at least some of the function(s) exhibited by the real *bona fide* antiporter proteins MrpA and MrpD. Furthermore, this experiment demonstrated that in this model system, Mrp complex formation was not required to exhibit antiporter function, since although the Mrp proteins and the Nuo proteins are homologous, it is not likely that NuoL or NuoN could form an assembled complex with the Mrp partner proteins.

3.4. The model system at pH 8.4

The salt sensitivity of the deletion strains was even more pronounced at alkaline pH (Fig. 2), and therefore the behavior of the model system was further investigated at pH 8.4. Expression of MrpA and MrpD could restore the growth properties of their respective deletion strain also at pH 8.4 (Fig. 6 and Table 2) but interestingly, only after a long lag phase. None of the complex I proteins could complement the deletion strains at this higher pH (Table 2). All these seemed to indicate that Mrp complex formation was essential for function at pH 8.4 but not at pH 7.4. To test this hypothesis the first part of the *mrp* operon, encoding the MrpABCD subcomplex, was cloned into the same expression vector, placing the

Table 2
Growth properties of *B. subtilis* deletion strains expressing antiporter proteins under different growth conditions.

	B. subtilis Δ*mrpA* at 80 mM Na^+			*B. subtilis* Δ*mrpD* at 60 mM Na^+		
	Max OD	*g* (min)	lag (h)	Max OD	*g* (min)	lag (h)
pH 7.4						
MrpA	1.83 ± 0.05	46	0	0.30 ± 0.02	na	na
MrpD	0.28 ± 0.03	na	na	1.89 ± 0.03	41	2
NuoL	1.74 ± 0.04	73	0	1.58 ± 0.02	115	2
NuoM	1.24 ± 0.02	155	0	0.40 ± 0.03	na	na
NuoN	0.43 ± 0.02	na	na	1.77 ± 0.03	76	2
pH 8.4						
MrpA	1.72 ± 0.03	64	3	0.12 ± 0.01	na	na
MrpD	0.13 ± 0.02	na	na	1.66 ± 0.04	56	6
NuoL	0.12 ± 0.01	na	na	0.14 ± 0.02	na	na
NuoM	0.13 ± 0.01	na	na	0.12 ± 0.01	na	na
NuoN	0.10 ± 0.02	na	na	0.14 ± 0.00	na	na
pH 6.5						
MrpA	1.70 ± 0.05	71	0	0.96 ± 0.02	110	2
MrpD	1.16 ± 0.02	104	1	1.81 ± 0.04	64	0
NuoL	1.61 ± 0.04	79	0	1.67 ± 0.04	98	2
NuoM	1.52 ± 0.04	91	0	1.62 ± 0.04	87	1
NuoN	1.65 ± 0.02	85	0	1.80 ± 0.02	89	0

lag = the length of the lag phase i.e. the time until onset of growth compared to that of the wild type *B. subtilis* 168 cells from the inoculation start point.
g = generation time (see Section 2.4).
na = not applicable. At very poor growth it is not meaningful to calculate a generation time or estimate lag time.

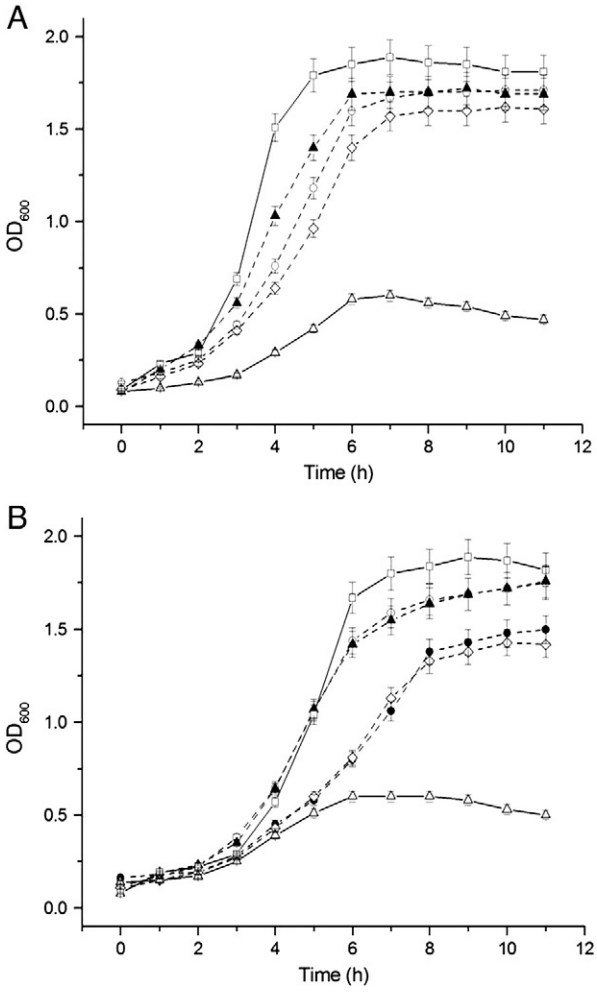

Fig. 5. *E. coli* complex I proteins restoring wild type properties to *B. subtilis* deletion strains (compare also Table 2). Complementation of *B. subtilis* Δ*mrpA* by NuoL is shown in panel A and complementation of *B. subtilis* Δ*mrpD* by NuoN in panel B. The cells were grown as in Fig. 3, at pH 7.4. In panel A, the growth of *B. subtilis* Δ*mrpA*/pVM8, expressing NuoL, is shown with dashed lines. At 100 mM NaCl the growth curve is marked by open diamonds, at 80 mM NaCl with open circles and at 60 mM NaCl with filled triangles. The *B. subtilis* Δ*mrpA*/pVM11 expressing the MrpA protein and grown at 100 mM NaCl (open squares) and *B. subtilis* Δ*mrpA*/pCW6, the empty expression vector, grown at 60 mM NaCl (open triangles) are shown for comparison. In panel B, the growth of *B. subtilis* Δ*mrpD*/pVM10 expressing NuoN is likewise illustrated with dashed lines. At 100 mM NaCl the growth curve is shown by open diamonds, at 80 mM NaCl as filled circles, at 60 mM NaCl as open circles and finally at 40 mM NaCl the growth progression is marked by filled triangles. *B. subtilis* Δ*mrpD*/pVM6 expressing the MrpD protein, and grown in 100 mM NaCl (open squares) and *B. subtilis* Δ*mrpD*/pCW6, containing the empty expression vector and grown at 40 mM NaCl (open triangles) are shown for comparison.

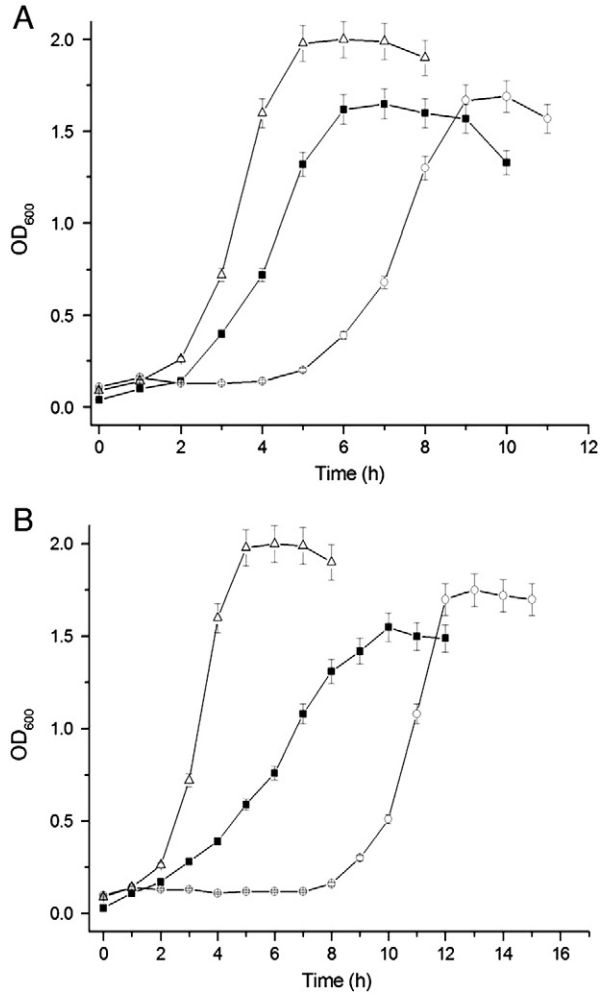

Fig. 6. Complementation of the deletion strains at pH 8.5. In panel A, the growth curves of *B. subtilis* Δ*mrpA*/pVM11 expressing only MrpA (open circles) and *B. subtilis* Δ*mrpA*/pVM7 expressing the MrpABCD subcomplex (filled squares) are compared to the growth properties of *B. subtilis* 168 (wild type, open triangles). The NaCl concentration was 80 mM. In panel B, *B. subtilis* Δ*mrpD*/pVM6 is complemented by MrpD alone (open circles) and by the MrpABCD subcomplex expressed from pVM7 (filled squares). The cells in panel B were grown at 60 mM NaCl. *B. subtilis* 168 (wild type, open triangles) grown at 60 mM NaCl is shown for comparison.

cytochrome *c* tag on MrpD. When this subcomplex was expressed, it resulted in a marked decrease in the lag phase in both deletion strains (Fig. 6A and B), corroborating the notion that the lag phase reflects the longer time needed for proper assembly of the complex when the subunits are expressed *in trans*. The generation times were longer in the cells expressing the MrpABCD subcomplex, 70 and 109 min in *B. subtilis* Δ*mrpA* and *B. subtilis* Δ*mrpD* respectively, compared to in cells expressing the respective single proteins (Table 2), even if the lag phase was abolished. This most likely reflects that the expressed MrpABCD subcomplex itself was immediately capable of antiporter activity at pH 8.4, but with lower activity/translocation efficiency than in the fully assembled MrpABCDEFG complex. The MrpABCD subcomplex from *Bacillus pseudofirmus* expressed in *E. coli* KNabc did however not show any antiporter activity [26]. Nevertheless, the alternative explanation, that the longer generation times simply reflect the rate of full complex assembly, seems much less likely. In such a scenario, one would expect a more gradual recovery also of the Δ*mrpA* cells expressing MrpA and the Δ*mprD* cells expressing MrpD, rather than the observed rapid growth after a long lag phase.

In addition, it was tested if a somewhat similar subcomplex could form from the complex I antiporter module NuoKLMN, by cloning the corresponding gene cluster into the same expression vector. The cells expressing these proteins together did not grow at pH 8.4 (not shown), indicating that no such functional subcomplex was formed. At pH 7.4, when comparing the expression of the NuoKLMN proteins in *B. subtilis* Δ*mrpA* to that of expression of NuoL alone, the generation time increased from 73 min (Table 2) to 110 min. In *B. subtilis* Δ*mrpD* the NuoKLMN expression resulted in a generation time of 102 min compared to only 76 min when NuoN was expressed alone (Table 2). The maximal cell density was also reduced in the cells simultaneously expressing the NuoKLMN and an extra 1-h lag phase appeared before the onset of growth (not shown). Taken together, this demonstrates that the NuoKLMN proteins do not form a functional subcomplex in this model system. The reduced growth efficiency observed reflects the extra protein expression work load imposed on these cells.

3.5. The model system at pH 6.5

To make the story complete, the properties of the model system was also explored at a more acidic pH than in Section 3.2. The two deletion strains *B. subtilis* Δ*mrpA* and *B. subtilis* Δ*mrpD* could be rescued by expressing their respective missing Mrp subunit from a plasmid also at pH 6.5, but the generation times were significantly longer at pH 6.5 compared to at pH 7.4 and compared to normal wild type cells grown at pH 6.5 (Fig. 7, Table 2). Surprisingly, the replacement was no longer completely exclusive. Some improvement of growth was seen also when expressing MrpD in *B. subtilis* Δ*mrpA* and MrpA in *B. subtilis* Δ*mrpD* strain, albeit with even longer generation times, and reaching only about half the normal maximal cell density. Mrp complex formation was clearly not needed for function at pH 6.5, since expression of Nuo proteins in the deletion strains could improve the growth. Interestingly, the Nuo proteins also ceased to show a marked specificity for Δ*mrpA* or Δ*mrpD* at pH 6.5. Although NuoL remains slightly better in rescuing the Δ*mrpA* strain and NuoN somewhat better in the Δ*mrpD* strain, all the Nuo proteins could rescue the deletion strains to an extent, and in all cases to a greater extent than when expressing MrpA in *B. subtilis* Δ*mrpD* and *vice versa* (Fig. 7A and B, Table 2). Taken together, this suggests a general mechanism, present in all the antiporter homologues, where the protonation of some residue(s) simultaneously affects the specificity of the respective subunit and at the same time reduces the translocation efficiency. Interestingly, pH dependent regulation of the cation selectivity was in fact recently observed in the homologous Pha antiporter system [51].

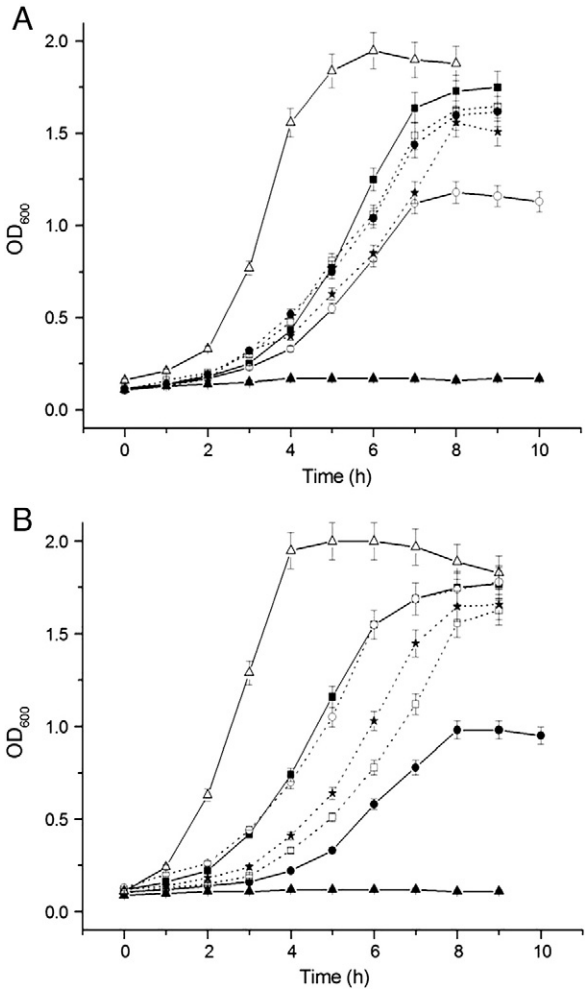

Fig. 7. Complementation of the deletion strains at pH 6.5. In panel A is shown *B. subtilis* Δ*mrpA* expressing the five different homologous proteins at pH 6.5 and 80 mM NaCl whereas in panel B, *B. subtilis* Δ*mrpD* expressing the same set of proteins at pH 6.5 and 60 mM NaCl is depicted. The growth curves of wild types cells (*B. subtilis* 168) at pH 6.5 and the respective NaCl concentrations are illustrated by open triangles in both panel A and panel B. Cells expressing the complex I proteins NuoL, NuoM and NuoN are shown with dotted lines in both panels. In panel A *B. subtilis* Δ*mrpA*/pVM11 that is expressing MrpA is show with filled squares, *B. subtilis* Δ*mrpA*/pVM8 expressing NuoL (open squares), *B. subtilis* Δ*mrpA*/pVM9 expressing NuoM (filled stars), *B. subtilis* Δ*mrpA*/pVM10 expressing NuoN (filled circles) and *B. subtilis* Δ*mrpA*/pVM6 expressing MrpD (open circles). The growth of *B. subtilis* Δ*mrpA* containing the empty expression vector pCW6 is shown as filled triangles. In panel B, the growth of *B. subtilis* Δ*mrpD*/pVM6 expressing MrpD is illustrated by filled squares, *B. subtilis* Δ*mrpD*/pVM8 expressing NuoL (open squares), *B. subtilis* Δ*mrpD*/pVM9 expressing NuoM (filled stars), *B. subtilis* Δ*mrpD*/pVM10 expressing NuoN (open circles) and *B. subtilis* Δ*mrpD*/pVM11 expressing MrpA (filled circles) to be compared with the growth curve of *B. subtilis* Δ*mrpD* that contain the expression vector pCW6 (filled triangles).

4. Discussion

This work has demonstrated that two of the antiporter-like proteins in complex I, NuoL and NuoN could complement the salt and pH sensitive growth phenotype of *B subtilis* MrpA and MrpD deletion strains and restore their wild type growth properties at pH 7.4 (Fig. 5). The two proteins did so in the combinations predicted from sequence, i.e. NuoL could rescue the Δ*mrpA* strain and NuoN could rescue the Δ*mrpD* strain. Therefore, not just primary sequence similarity, but an actual functional similarity must exist between these proteins. The antiporter-like complex I subunit NuoM, on the other hand, could not complement any of the deletions strains to that extent. We can of course not rule out that NuoM could be active under some condition that has not been tested here. Alternatively, NuoM may more easily slip into an inactive conformation when expressed as a single protein outside complex I. Nevertheless, at acidic pH, when all the homologous antiporter-like proteins showed some activity, NuoM behaved no different than the others (Fig. 7).

In the *B. subtilis* model system implemented in this work the MrpA and MrpD proteins were shown to function independently, without the formation of an MrpABCDEFG complex, at pH 7.4 or more acidic pH, but required complex formation to function at more alkaline pH. Previous work on the Mrp antiporter system has converged on the conclusion that the Mrp proteins are only functional as a complex [7,24,25]. MrpA and MrpD expressed in the *E. coli* KNabc strain, that has been deleted for endogenous antiporters, showed no detectable antiporter activity. An MrpA-D subcomplex expressed in the same *E. coli* strain was also devoid of antiporter activity [26,52]. There is in fact only one previous example of a MrpA/D type protein, one from a polyextremophilic bacterium, that exhibited antiporter activity when expressed as a single protein in *E. coli* KNabc [53]. The Mrp proteins used in the former experiments were from an alkalophile bacterium that may have more obligatory requirements for Mrp complex formation. The major difference between the *E. coli* KNabc experimental system and the *B. subtilis* deletion strains used here is however that the other Mrp proteins in the complex are absent in the former system. In the *B. subtilis* deletion strains only one gene, encoding either MrpA or MrpD, has been excised from the chromosome. The bleomycin resistance gene used for the deletions was inserted in the reverse direction to facilitate RNA polymerase read-through, and minimize the risk of affecting the expression of downstream genes. Since expression of either of the missing subunits MrpA or MrpD could restore growth at pH 8.4 after a lag phase allowing complex assembly to take place, it can be safely concluded that the other subunits are indeed being produced in each *B. subtilis* deletion strain. To account for why MrpA could never replace MrpD and *vice versa* at pH 7.4, when Mrp complex formation was not needed for function, the simplest explanation would be that each protein is responsible for the translocation of only one ion species. Therefore, both MrpA and MrpD must be present for antiporter activity to occur. This would also explain why the individual proteins showed no antiporter activity when expressed in *E. coli* KNabc.

It was previously observed that MrpA is more similar to NuoL whereas MrpD more closely resemble NuoM and NuoN [11]. Since NuoL from *E. coli* was previously shown to conduct Na^+ [16,54], and the primary sequence of NuoL is more similar to that of MrpA [11] we may postulate that MrpA conducts Na^+ whereas MrpD conducts H^+ in such a scenario. When the external medium is more acidic or neutral, acidification of the bacterial interior through the MrpBCD/EFG subcomplex can drive Na^+ extrusion through MrpA or NuoL without complex formation, allowing the bacteria to tolerate NaCl in the growth medium. Acidification of the interior can also take place through the plasmid expressed MrpD or NuoN, thereby causing Na^+ extrusion through the MrpABC/EFG subcomplex. This scenario would explain the discrepancy between the experiments done in *E. coli* KNabc, where the expressed single MrpA or MrpD showed no antiporter activity [26,34], and the experiments done in *B. subtilis* Δ*mrpA* and *B. subtilis* Δ*mrpD* where the expressed single MrpA and MrpD show activity, since activity requires that the other partner proteins are present, even if complex formation is optional. At alkaline pH, an energy input and presumably a conformational coupling is needed to carry out the reaction, and therefore only a fully assembled MrpABCD/EFG complex can carry out this work. The NuoL, NuoM and NuoN proteins are not expected to be able to form a complex with the Mrp antiporter proteins, and therefore it is not surprising that no complementary activity could be obtained with the any of the Nuo proteins at pH 8.5 (Table 2).

The postulated single ion transporter scenario is somewhat complicated by the observations made at pH 6.5. Clearly, an excess of external protons did not just facilitate Na^+ extrusion without Mrp complex formation as suggested above, but it also lead to some promiscuous activity by all the Nuo proteins. Even the MrpA and D proteins were no longer completely mutually exclusive at pH 6.5 (compare Figs. 4 and 7). Nevertheless, since the behavior of all the five proteins was affected by the lowered pH in the growth medium, it could be possible that the protonation of some conserved amino acid residue(s) might affect the actual ion specificity of the respective subunit. When the individual proteins were no longer specific for one ion, that would be expected to also affect the overall efficiency of the antiporter complex. Notably, somewhat longer generation times were indeed observed for *B. subtilis* Δ*mrpA* expressing MrpA and *B. subtilis* Δ*mrpD* expressing MrpD at pH 6.5 (Table 2). It should also be emphasized that the generation time of wild type *B. subtilis* is essentially the same in the pH range 6.5–8.5. The unspecific activity and longer generation times seen at pH 6.5 in the deletion strains expressing MrpA and MrpD can therefore be correlated to conditions when the polypeptides are not assembled into a Mrp complex. Since many acidic residues have been shown to be important for function [32,34], the protonation that affected ion specificity in the single subunits may still modulate activity also in the respective assembled protein complexes.

To reiterate, although alternative explanations involving complicated regulatory scenarios are still possible, the simplest explanation to all the previous observations is that the individual MrpA and MrpD proteins are single ion transporters, that together form an Mrp antiporter complex. Previous experiments have consistently shown how both MrpA and MrpD must be present for antiporter function to occur. We have demonstrated that homologous complex I subunits could rescue bacterial strains lacking either MrpA or MrpD, a complementation that must have occurred without any Mrp complex formation. If NuoN was in itself an antiporter, we must envision a mechanism that would allow this antiporter to function well when expressed in *B. subtilis* Δ*mrpD* at pH 7.4 but make it shut down when expressed in *B. subtilis* Δ*mrpA* at the same pH (Fig. 5, Table 2). The same argument holds for the fact that expression of MrpA could not compensate for the loss of MrpD and *vice versa* (Fig. 3). Therefore, it can be assumed that complex I contain three membrane-spanning single ion transporter proteins. Most probably, NuoL comprises a Na^+ transporter and NuoN a H^+ transporter. A number of site directed mutations in NuoL were recently shown to affect the proton pumping efficiency of complex I in *E. coli* membrane vesicles [33]. This does not necessarily contradict our proposed role for NuoL, since the Na^+ and H^+ transport activities probably are tightly coupled in the assembled complex I, and therefore are expected to depend on each other. Unfortunately, no decisive function for NuoM could be deduced from this work. The many site-directed mutations in NuoM that have been demonstrated to affect complex I function [21–23] makes it improbable that NuoM should have a mere structural role. In addition, NuoM was capable of ion translocation under the promiscuous conditions (Fig. 7). Furthermore, we were not able to learn anything about the function of NuoL, NuoM and NuoN at pH 8.4 since the Mrp complex formation requirements restrict the measuring range of our model system. Taken together, NuoM is probably also active in complex I, and based on the prior bioinformatic results [11], it is most likely that NuoM is also a H^+ transporter. The implications for the complex I functional mechanism are therefore that one Na^+ channel and two H^+ channels are present. This is in agreement with recent work by Manuela Pereira and coworkers, showing that the presence of Na^+ increased the proton transport of *Rhodothermus marinus* complex I [55]. The complex I site for proton translocation postulated by Pereira would correspond to NuoN whereas the site for Na^+/H^+ antiporter postulated by Pereira would correspond to NuoL and NuoM.

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