Structure and function of the C-terminal domain of MrpA in the Bacillus subtilis Mrp-antiporter complex – The evolutionary progenitor of the long horizontal helix in complex I

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Abstract
MrpA and MrpD are homologous to NuoL, NuoM and NuoN in complex I over the first 14 transmembrane helices. In this work, the C-terminal domain of MrpA, outside this conserved area, was investigated. The transmembrane orientation was found to correspond to that of NuoJ in complex I. We have previously demonstrated that the subunit NuoK is homologous to MrpC. The function of the MrpA C-terminus was tested by expression in a previously used Bacillus subtilis model system. At neutral pH, the truncated MrpA still worked, but at pH 8.4, where Mrp-complex formation is needed for function, the C-terminal domain of MrpA was absolutely required.

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1. Introduction

Complex I or NADH:quinone oxidoreductase is the last enzyme in the classical mitochondrial respiratory chain for which high resolution structural information became available [1–4]. The electron transfer from the NADH binding site on the NuoE/51 kDa subunit via a series of FeS clusters to the quinone binding site in the NuoD/49 kDa subunit [5], is coupled to proton pumping across the membrane domain with a 4H+/2e−/C0 stoichiometry [6]. However, the actual coupling mechanism between the two activities remains poorly understood. The membrane domain of complex I is dominated by three large, conserved subunits. In addition to being similar to each other [7], the primary sequence of NuoL/ND5, NuoM/ND4 and NuoN/ND2 was found to be homologous to two other proteins, MrpA and MrpD from the Mrp-antiporter complex [8]. In addition, MrpA/NuoL and MrpD/NuoM and N form two distinct phylogenetic clusters [9], showing that the protein family is composed of two types of homologous polypeptides with somewhat different functions. This was later corroborated by functional comparisons using a model system where plasmid-encoded MrpA or NuoL, could rescue Bacillus subtilisΔmrpA but not B. subtilisΔnuoL and NuoN revealed an almost perfect structural overlap with each other over the first 14 TM helices [1]. Only the most distally located subunit, NuoL, contains an additional C-terminal segment not present in the other proteins. This domain was also the largest surprise from the structure, and revealed as a long horizontal helix originating from NuoL in the distal end of the membrane domain and reaching back all the way towards the proximal, promontory domain. This domain consists of a predicted transmembrane segment (helix 15), a long horizontal helix, followed by a final transmembrane helix (helix 16). This intriguing structure, found in both prokaryote and eukaryote complex I [1,3], was named “coupling rod” or “piston”, and initially envisioned to provide the means for the long range conformational changes needed to drive the redox coupled proton pump. The function of the C-terminal extension in NuoL has since then been addressed in some recent papers. Removal of the domain affected the proton pumping efficiency of the purified and liposome-reconstituted enzyme. Mutations of protonatable residues (particularly Asp563) in the Escherichia coli
long horizontal helix also affected H'\(e^-\) stoichiometry [11]. Other studies showed that truncation of NuoL greatly affected the stability of the complex in vivo [12]. To further scrutinize the proposed piston-like function, a subsequent analysis of both point mutations, insertions and substitutions demonstrated that although many of the manipulations affected complex I stability, by altering the “piston”, it still retained the normal H'\(e^-\) stoichiometry [13]. Therefore, it was concluded that the NuoL long horizontal helix is important for stability and formation of a mechanical connection, but its role in energy transduction remains uncertain.

The structural information from complex I can be used to make a model of the homologous parts of the MrpA and MrpD subunits of the Mrp-antiporter [14]. It was noticed that NuoK and MrpC are homologous proteins [15], which corroborated the notion that a whole antiporter module was recruited to complex I and NuoL, NuoM and NuoN were not simply the result from triplication of one primordial gene [18]. The most recent high resolution structure revealed that the NuoH subunit has an antiporter-fold too, one primordial gene [18]. The most recent high resolution structure revealed that the NuoH subunit has an antiporter-fold too, one primordial gene [18].

Table 1

| Bacteria       | Relevant properties             | Reference or source |
|----------------|---------------------------------|---------------------|
| E. coli XL1-Blue | recAI, endAI, gyrA56, thi, hsdR17, supE44, relAI (lac) | Promega |
| E. coli JM109   | endAI, ginV44, thi-1, relAI, gyrA56, recA1, mcrBp B[| lacproAB] e14-[F0 traD36 proA Bp lacIq lac2DM15] hsdR17 (r_m-2) | Promega |
| B. subtilis 168A | Wild type, (type train), trpC2   | C. subtilis Genetic Stock Center |
| B. subtilis ΔmrpA | ΔmrpA ble'                   |                      |
| Plasmids       |                                 |                     |
| pEC86          | ccm operon, Cm\(^R\)             |                     |
| pVM4h          | mrpA fused with cca\(A^-\)6xCAT, Amp\(^R\) |                     |
| pVM4[P474]     | mrpA truncated at the codon encoding P474 and fused with cca\(A^-\)6xCAT, Amp\(^R\) | This work |
| pVM4[K679]     | mrpA truncated at the codon encoding K679 and fused with cca\(A^-\)6xCAT, Amp\(^R\) | This work |
| pVM4[V734]     | mrpA truncated at the codon encoding V734 and fused with cca\(A^-\)6xCAT, Amp\(^R\) | This work |
| pVM4[P747]G9C  | mrpA carrying mutation G9C, truncated at the codon encoding P747 and fused with cca\(A^-\)6xCAT, Amp\(^R\) | This work |
| pMCh           | nuoM fused with cca\(A^-\)6xCAT, Amp\(^R\) | This work |
| pMM(P474)      | mrpA with first 4 codons replaced by first 4 codons of nuoM, truncated at the codon encoding P474 and fused with cca\(A^-\)6xCAT, Amp\(^R\) | This work |
| pMM(K679)      | mrpA with first 4 codons replaced by first 4 codons of nuoM, truncated at the codon encoding K679 and fused with cca\(A^-\)6xCAT, Amp\(^R\) | This work |
| pMM(V734)      | mrpA with first 4 codons replaced by first 4 codons of nuoM, truncated at the codon encoding V734 and fused with cca\(A^-\)6xCAT, Amp\(^R\) | This work |
| pMM4           | mrpA with first 4 codons replaced by first 4 codons of nuoM and fused with cca\(A^-\)6xCAT, Amp\(^R\) | This work |
| pCW6           | Cm\(^R\)                       | Claes von Wachenfeldt |
| pVM4h          | mrpA fused with cca\(A^-\), Cm\(^R\) | [18]               |
| pMrpAtr        | mrpA truncated at the codon encoding I472 and fused with cca\(A^-\), Cm\(^R\) | This work |
| pVM7           | mrpABCD fused with cca\(A^-\) at mrpD, Cm\(^R\) |                     |
| pMrpABCD       | mrpABCD with mrpA truncated at the codon encoding I472 and fused with cca\(A^-\) at mrpD, Cm\(^R\) | This work |
| Primers        | Primer sequence               |                     |
| MrpAtr-fow     | 5'\-GGCGCCTGAGCTATCTGCGG-3'   |                     |
| MrpAtr-P747-rev| 5'\-AGCGTCTCTATACGGCTGCGAC-3' |                     |
| MrpAtrK679-rev | 5'\-TTTGGTTTTTACAGCTCATGCTGC-3' |                     |
| MrpAtrV734-rev | 5'\-CAACTTACATAATTCTATCGT-3' |                     |
| MrpAG6A-fow    | 5'\-AGCGTCTCTATACGGCTGCGAC-3' |                     |
| MrpAG6A-rev    | 5'\-TTTGGTTTTTACAGCTCATGCTGC-3' |                     |
| MrpABCD-T13-fow| 5'\-GTGTGTTTACATGCTGAG-3'    |                     |
| NuoM-Blunt-C   | 5'\-GGCGCCTGAGCTATCTGCGG-3'   |                     |
| MrpA-cW6-up    | 5'\-GGCGCCTGAGCTATCTGCGG-3'   |                     |
| MrpB-cW6-down  | 5'\-CTTACACGGCTGAGCTATCTGCGG-3' |                     |
| MrpA-trV734-rev| 5'\-AGCGTCTCTATACGGCTGCGAC-3' |                     |

2. Materials and methods

2.1. Molecular biology

For fusion protein expression, E. coli JM109, pEC86 were grown microaerophilicly in LB media containing 0.5% NaCl, 50 μM IPTG for 40 hours at 30 °C, 200 rpm. For solid media, 1.5% agar was added. B. subtilis strains were kept on TBAB plates (Difco). Antibiotics were added in the following concentrations when appropriate: 100 μg/ml ampicillin (Sigma), 12.5 μg/ml chloramphenicol for E. coli and 5 μg/ml chloramphenicol (Duchefa) for B. subtilis. The primers were synthesized by Invitrogen. Standard Molecular Biology techniques were from Fermentas or New England Biolabs. The PCRs were performed using High Fidelity Phusion Hot Start II DNA polymerase (Finnzymes). DNA sequencing reactions were carried out using Big Dye™ (Applied Biosystems) at the Biomolecular Resource Facility, Lund University.

2.2. Transmembrane topology prediction

The primary sequences were collected from the protein sequence database UniProt. The multiple sequence alignments were done using CLUSTALW [16]. The structure-based sequence
alignments were done using the high resolution crystal structure [14]. The transmembrane topology was predicted by HMMTOP, TMHMM from the Center for Biological Sequence Analysis, Department of Systems Biology, Technical University of Denmark, TopPred [17] and TMPred provided by EMNet with their default values.

2.3. Construction of the fusion proteins with cytochrome c

The plasmids pVM4(P474) encoding MrpA truncated at P474, pVM4(K679) encoding MrpA truncated at K679 and pVM4(V734) encoding MrpA truncated at V734 were generated by an amplification of the plasmid pVM4-h encoding full length MrpA from B. subtilis C-terminally fused with a cyt c550-His-tag [18]. The PCR products were done using one downward primer, MrpAtr-forw, and three different upward primers for the different mrpA truncations: MrpAtrP474-rev, MrpAtrK679-rev and MrpAtrV734-rev (Table 1). The PCR products were self-circularized and used for transformation of E. coli XL1-Blue. The transformants were selected on LB-agar plates containing ampicillin and sequenced over the truncation points. Subsequently, E. coli JM109 harboring pEC86 [19] was transformed with the constructed plasmids.

The plasmid pVM4(P474)/G9C carrying a G9C mutation in the truncated mrpA gene was constructed by amplifying the plasmid pVM4(P474) using two primers: MrpAG9C-forw and MrpAG9C-rev (Table 1). Subsequent ligation, transformation and selection were done as before.

2.4. Construction of the expression plasmids encoding full and truncated MrpA

The plasmid pMCh encoding NuoM from E. coli fused with cyt c550-His-tag [18] was used for a partial replacement of nuoM gene by mrpA gene. The mrpA sequence encoding MrpA lacking the first four amino acids and truncated at P474 was amplified from the genomic DNA of B. subtilis by using the primers MrpA-Blunt-T13-forw and MrpAtrP474-rev (Table 1). The vector pMCh was amplified by using the primers Cytc-Blunt-G1-forw and NuoM-Blunt-C12-rev (Table 1) omitting the nuoM gene but leaving the first four codons encoding MLLP of NuoM. The two PCR fragments having only blunt ends were ligated together, used for transformation and selected as before. The construct was sequenced over the two fusion points and named pMMa(P474).

Subsequently, the constructs pMMa(K679), pMMa(V734) and pMMA were made. Fragments MrpA(K679)-cytc, MrpA(V734)-cytc and mrpA-cytc, from pVM4(K679), pVM4(V734) and pVM4h, respectively, were generated by restriction with FseI cutting in mrpA and PpMUL cutting in cytochrome c gene, and inserted into pMMA(P474) cleaved by the same enzymes. The constructs were verified by sequencing over the promoter and the entire fusion gene region and used for transformation of the expression strain E. coli JM109 harboring pEC86 [19].

2.5. Construction of C-terminally truncated MrpA and MrpABCD

For construction of pMrpAtr, the entire pVM11 encoding mrpA from B. subtilis in pCW6 was amplified omitting the mrpA gene part encoding the C-terminus from I472 by using the primers MrpAtr-pCW6-up and MrpAtr-pCW6-down (Table 1). For truncation of the C-terminal domain of MrpA expressed in MrpABCD complex, the plasmid pVM7 containing the mrpABCD gene, was amplified omitting the same part of mrpA gene as before by using the primers MrpABCD-pVM7-up and MrpABCD-pVM7-down (Table 1) The PCR products were self-circularized and used for transformation of E. coli JM109. The constructs were sequenced over the truncation point. The B. subtilis ΔmrpA strain was subsequently transformed with the constructed plasmids.

2.6. Production of the cytochrome c fusion proteins

The full length and truncated MrpAcytH, MrpA(G9C)cytH, MMrpAcytH were produced in E. coli JM109 constitutively coexpressing the ccm operon, which is responsible for heme insertion and cytochrome c maturation, from pEC86 (kindly provided by Linda Thöny-Meyer). The cell membranes were isolated as described previously [18]. Protein concentration was determined by BCA Protein Assay Kit (Pierce) with bovine serum albumin (Sigma), as standard. The proteins were purified using a HisTrap column (GE Healthcare) as before [20]. Proteins were concentrated using Millipore Amicon Ultra, MWCO 30000.

2.7. Western blot and heme staining

SDS-PAGE was performed as described by Neville using 10% polyacrylamide gels [21]. The transfer to PVDF membrane (Millipore) was done according to the manufacture’s instructions. The immunodetection was done as described previously [20] using anti-cyt c550 rabbit antibodies, as the primary antibody (kindly provided by Lars Hederstedt), and alkaline phosphatase linked goat-anti rabbit antibodies, as the secondary antibody. The PVDF membrane was developed with ECF (GE Healthcare). Activities were monitored as a blue fluorescence at 530 nm using a Bio-Rad ChemiDoc MP imaging system. For heme staining, the peroxidase activity was visualized directly in the gel by the method of Kashino et al. [22] using 3,3-diaminobenzidine tetrahydrochloride, as substrate.

2.8. Optical spectroscopy

Optical spectra of the cyt c550-tagged proteins were recorded using a Shimadzu UVPC 2100 spectrophotometer. Using the extinction coefficient of cytochrome c550 = 24 mM-1 cm-1 [23], concentrations of the fusion proteins were calculated [20].
Fig. 2. A and B. Structure-based sequence alignments of the C-terminal domain of MrpA with NuoL (A) and NuoJ (B) (see also Fig. 1). The sequences are (from top to bottom): Bacillus subtilis [Q9K2S2], Staphylococcus aureus [Q0Q2K0], Corynebacterium glutamicum [Q8NM51], Escherichia coli [P33607], Thermus thermophilus [Q56227], Bacillus cereus [B3YZU6]. Orientation of the predicted transmembrane segments is indicated by letters: i – for ‘inside’ and o – for ‘outside’. The conserved residues are in bold. The positions, where cytochrome c550 domain was fused to MrpA, are indicated by arrows. (A) The first part of the C-terminal domain: positions of the transmembrane segments of MrpA were in good agreement with the known structure of NuoL [14], and are highlighted by gray boxes. The helix numbering on top of the sequence is referring to MrpA and on bottom to NuoL. Amino acids that have been mutated in NuoL from E. coli and reported in the literature [11,25] are marked by black dots. Those that showed a significant effect on complex I activity or proton pumping capacity when mutated are surrounded by squares. The long helix in MrpA from B. subtilis, region L535–R554, is approximately half of the size of the long horizontal helix in NuoL from E. coli, region A538–L583 (19–23 vs 41–46 amino acids in the selected organisms, respectively). (B) The second part of the C-terminal domain: position of the transmembrane segments in MrpA were in good agreement with the known structure of NuoJ [14], and are highlighted by gray boxes. Amino acids that have been mutated in MrpA from B. subtilis and reported in the literature [30] are marked by stars and those in NuoJ from E. coli [31–32] are marked by dots. Amino acids that showed a significant effect on complex activity when mutated are surrounded by squares.
2.9. Growth studies in B. subtilis

All growth studies were done as described previously [10].

3. Results and discussion

The predicted transmembrane topology of the MrpA polypeptide is schematically shown in Fig. 1. The first 14 TM helices (Fig. 1, A) correspond to the domain conserved in all members of the protein family. The C-terminal domain of MrpA can be further divided in two parts: the first two transmembrane helices with a long helix in between, corresponding to the part conserved in NuoL (Fig. 1, B) and the second part comprising five predicted TM helices that we postulated correspond to NuoJ in complex I (Fig. 1, C [24]). As seen in Fig. 2, there are a few conserved elements in this area, but the overall sequence similarity is low. To assess the function of the long horizontal helix in NuoL, point mutations as well as more detailed insertions and other modifications to alter the putative “piston” rigidity have been made by other groups [12–13,25]. The size of the structural element found in NuoL, but not in NuoM and N, is ranging from 57–129 amino acids in complex I, whereas the corresponding sequence in MrpA is generally smaller, ranging from 41 to 54 amino acids long. The size difference is the most prominent in the area shown in Fig. 2A, where the central part of the MrpA long helix is about half the length (15–23 amino acids in MrpA’s) of that seen in the equivalent stretch (A538–L583) in the structure of E. coli (46 amino acids). The length of this

Table 2
Growth properties of B. subtilis deletion strain, expressing antiporter-like proteins under different growth conditions.

| B. subtilis ΔmrpA at 80 mM Na⁺ | Max OD g (min) |
|-------------------------------|----------------|
| MrpA                         | 1.72 ± 0.03    |
| MrpAt                        | 1.64 ± 0.02    |
| MrpA                         | 1.84 ± 0.06    |
| MrpAtr                       | 1.49 ± 0.05    |
| MrpABCD                      | 1.78 ± 0.02    |
| MrpAtrBCD                    | 1.43 ± 0.02    |
| At pH 7.4                     | 1.76 ± 0.03    |
| MrpA                         | 0.17 ± 0.02    |
| MrpABCD                      | 1.65 ± 0.02    |
| MrpAtrBCD                    | 0.16 ± 0.03    |

Fig. 3. The four different cytochrome c fusion proteins in purified and concentrated fractions detected by immunodetection with anti-cyt c<sub>550</sub> antibodies (Panel A) and heme staining (Panel B). The calculated masses of the proteins are: 63.8 kDa for MMrpA[P474]cytH in Lane 1, 86.8 kDa for MMrpA[K679]cytH in Lane 2, 92.7 kDa for MMrpA[V734]cytH in Lane 3 and 97.1 kDa for MMrpA[acetoc]cytH in Lane 4 (pointed by arrows). NuoMcytH, with a calculated mass of 67.2 kDa, was used as a control to account for the somewhat anomalous migration of membrane proteins in gels. The last lane in Panel A and the first lane in Panel B contained 5 μg of NuoMcytH, others contained 50 μg of membrane protein. Both gels contain some degradation products however occurring only in the holo-cytochrome-proteins.

Fig. 4. Growth properties of the B. subtilis ΔmrpA deletion strain at pH 7.4 and 80 mM Na⁺. MrpA expressed in trans from a plasmid restores the wild type growth properties, but expression of MrpD does not improve the growth. Expression of MrpA lacking the C-terminal domain resulted in a moderate growth improvement compared to the full length MrpA (A). Expression of MrpABCD from a plasmid also results in wild type growth properties, whereas truncated MrpA expressed within the operon context (B) behaved essentially as the truncated MrpA expressed alone.
structural elements, corresponding to HLα plus HLβ in the E. coli structure [14], are in the range of 35–52 amino acids in NuoL sequences in general. This probably reflects that MrpA has only one homologous partner protein, MrpD, whereas long horizontal helix in NuoL must embrace both NuoM and NuoN subunits.

The predicted topology of MrpA was then tested experimentally using the cytochrome c Petra fusion method described previously [20]. Briefly, the heme insertion apparatus needed for covalent attachment of heme to the CxxCH motif in c-type cytochromes is only present in the E. coli periplasm [19,26]. Therefore, a holo-cytochrome can only be formed when the membrane protein to be tested has the C-terminus on the periplasmic side of the membrane when the fusion protein is expressed in E. coli, whereas otherwise the apo-cytochrome can be detected using anti-cytochrome c Petra antibodies. Therefore, the cytochrome-tagging strategy is applicable to determine the transmembrane topology of membrane proteins [20] and is also useful to stabilize and enhance the expression of some proteins [18].

It was noted earlier that the B. subtilis MrpA mRNA form a stem-loop structure immediately downstream of the GTG start codon of mrpA which interferes with translation using an inducible promoter instead of the naturally regulated expression. A synonymous mutation (G9 to C) that previously was used to avoid the formation of the secondary structure and allow protein synthesis [27], was however not sufficient to achieve reasonably high MrpA protein production in E. coli (not shown). Therefore, a novel construct containing the upstream region and four coding residues of the NuoM, a construct that normally allows high expression levels [18], was tested in trans (not shown). Therefore, a novel construct containing the upstream region and four coding residues of the NuoM, a construct that normally allows high expression levels [18], was made. Yet, the expressed amount of MMrpA(K679)cytH corresponding to the fully conserved part of MrpA (Fig. 1A), was only 30% of what we normally produce of NuoM. Still, it definitely corresponds to the fully conserved part of MrpA (Fig. 1A), was only 30% of what we normally produce of NuoM. Still, it definitely corresponds to the fully conserved part of MrpA (Fig. 1A), was only 30% of what we normally produce of NuoM. Still, it definitely corresponds to the fully conserved part of MrpA (Fig. 1A), was only 30% of what we normally produce of NuoM. Still, it definitely corresponds to the fully conserved part of MrpA (Fig. 1A), was only 30% of what we normally produce of NuoM. Still, it definitely corresponds to the fully conserved part of MrpA (Fig. 1A), was only 30% of what we normally produce of NuoM. Still, it definitely corresponds to the fully conserved part of MrpA (Fig. 1A), was only 30% of what we normally produce of NuoM. Still, it definitely corresponds to the fully conserved part of MrpA (Fig. 1A), was only 30% of what we normally produce of NuoM. Still, it definitely corresponds to the fully conserved part of MrpA (Fig. 1A), was only 30% of what we normally produce of NuoM. Still, it definitely corresponds to the fully conserved part of MrpA (Fig. 1A), was only 30% of what we normally produce of NuoM. Still, it definitely corresponds to the fully conserved part of MrpA (Fig. 1A), was only 30% of what we normally produce of NuoM. Still, it definitely corresponds to the fully conserved part of MrpA (Fig. 1A), was only 30% of what we normally produce of NuoM. Still, it definitely corresponds to the fully conserved part of MrpA (Fig. 1A), was only 30% of what we normally produce of NuoM. Still, it definitely corresponds to the fully conserved part of MrpA (Fig. 1A), was only 30% of what we normally produce of NuoM. Still, it definitely corresponds to the fully conserved part of MrpA (Fig. 1A), was only 30% of what we normally produce of NuoM. Still, it definitely corresponds to the fully conserved part of MrpA (Fig. 1A), was only 30% of what we normally produce of NuoM. Still, it definitely corresponds to the fully conserved part of MrpA (Fig. 1A), was only 30% of what we normally produce of NuoM. Still, it definitely corresponds to the fully conserved part of MrpA (Fig. 1A), was only 30% of what we normally produce of NuoM. Still, it definitely corresponds to the fully conserved part of MrpA (Fig. 1A), was only 30% of what we normally produce of NuoM. Still, it definitely corresponds to the fully conserved part of MrpA (Fig. 1A), was only 30% of what we normally produce of NuoM. Still, it definitely corresponds to the fully conserved part of MrpA (Fig. 1A), was only 30% of what we normally produce of NuoM. Still, it definitely corresponds to the fully conserved part of MrpA (Fig. 1A), was only 30% of what we normally produce of NuoM. Still, it definitely corresponds to the fully conserved part of MrpA (Fig. 1A), was only 30% of what we normally produce of NuoM. Still, it definitely corresponds to the fully conserved part of MrpA (Fig. 1A), was only 30% of what we normally produce of NuoM. Still, it definitely corresponds to the fully conserved part of MrpA (Fig. 1A), was only 30% of what we normally produce of NuoM. Still, it definitely corresponds to the fully conserved part of MrpA (Fig. 1A), was only 30% of what we normally produce of NuoM. Still, it definitely corresponds to the fully conserved part of MrpA (Fig. 1A), was only 30% of what we normally produce of NuoM. Still, it definitely corresponds to the fully conserved part of MrpA (Fig. 1A), was only 30% of what we normally produce of NuoM. Still, it definitely corresponds to the fully conserved part of MrpA (Fig. 1A), was only 30% of what we normally produce of NuoM. Still, it definitely corresponds to...
was truncated at position 1472 (Fig. 2A), both in the single MrpA subunit and in MrpABCD sub-complex, and expressed in *B. subtilis* ΔMrpA. The deletion strain was grown at different pH and the cells were challenged with 80 mM Na+, conditions that were tried out earlier [10]. At pH 7.4 the bacteria expressing the truncated MrpA were growing a bit slower than the wild type, with a generation time of 97 min compared to 46 min for the full length protein. Likewise, the bacteria expressing MrpABCD from a plasmid were a bit slower than expressing MrpA alone, irrespectively if the MrpA was truncated or not (Table 2, Fig. 4). The slightly longer generation times reflect the heavier load to express four extra proteins instead of just one. At pH 8.4, the MrpA expressing deletion strain showed a long lag-phase that is not seen in the MrpABCD case. This was observed previously [10], and was interpreted such that Mrp-complex formation is required for the antiporter-complex to work at more alkaline pH. Therefore, MrpABCD expressed together can start to operate immediately, whereas a subunit expressed alone from a plasmid will need to be incorporated into a newly synthesized chromosomally encoded protein-complex, resulting in the observed lag-phase. This interpretation also fitted the observation that complex I subunits could substitute for MrpA or MrpD at neutral pH, but never at pH 8.4. Interestingly, at pH 8.4, the truncation of MrpA had a drastic effect on growth. Neither the MrpAtr alone nor the MrpAtrBCD could rescue the *B. subtilis* ΔMrpA strain (Table 2, Fig. 5). This demonstrates the structural importance of the long horizontal helix and the whole C-terminal domain also in the Mrp-complex. This further implies that the conserved part of MrpA, a subunit presumably responsible for ion conduction, is working in spite of the truncation, albeit a bit slower, whereas the C-terminal domain is essential for Mrp-complex formation and growth at pH 8.4. Unfortunately, no real high resolutions structural information is available for the Mrp-antiporter complex, but taken together, we can propose substantial structural similarities between the two (Fig. 6).

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