



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 $4H^+/2e^-$ 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 subtilis* Δ mrpA but not *B. subtilis* Δ mrpD, whereas the opposite was seen for plasmid-encoded MrpD and NuoN [10].

The structures of three complex I proteins NuoL, NuoM 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*

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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, although any primary sequence similarity has been obliterated [4]. Since MrpA is the evolutionary progenitor of NuoL, in this work the structure and function of the C-terminal domain of MrpA, projecting beyond the universally conserved domain, was investigated. Interestingly, not only the long horizontal helix, but also the NuoJ subunit in complex I originates in this part of MrpA. The function of the antiporter-domain of complex I could also depend on its evolutionary relationship with Mrp. This issue was addressed by truncating the MrpA subunit, and investigating the

function in the *B. subtilis* strains deleted for *mrpA*, that was previously utilized as a model system [10].

2. Materials and methods

2.1. Molecular biology

For fusion protein expression, *E. coli* JM109, pEC86 were grown microaerophilically in LB media containing 0.5% NaCl, 50 μ M 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 μ g/ml ampicillin (Sigma), 12.5 μ g/ml chloramphenicol for *E. coli* and 5 μ g/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

Table 1
Bacteria, plasmids and primers used in this work.

Bacteria	Relevant properties	Reference or source
<i>E. coli</i> XL1-Blue	recA1, endA1, gyrA96, thi, hsdR17, supE44, relA1 (lac)	Promega
<i>E. coli</i> JM109	endA1, glnV44, thi-1, relA1, gyrA96, recA1, mcrBp D(lacproAB) e14-[F0 traD36 proABp lacIq lacZDM15] hsdR17(r_{K-m_K})	Promega
<i>B. subtilis</i> 168A	Wild type, (type train), trpC2	Bacillus Genetic Stock Center
<i>B. subtilis</i> Δ mrpA	Δ mrpA ble ^f	[10]
Plasmids		
pEC86	<i>ccm</i> operon, Cm ^R	[26]
pVM4h	<i>mrpA</i> fused with <i>cccA'</i> -6xCAT, Amp ^R	This work
pVM4(P474)	<i>mrpA</i> truncated at the codon encoding P474 and fused with <i>cccA'</i> -6xCAT, Amp ^R	This work
pVM4(K679)	<i>mrpA</i> truncated at the codon encoding K679 and fused with <i>cccA'</i> -6xCAT, Amp ^R	This work
pVM4(V734)	<i>mrpA</i> truncated at the codon encoding V734 and fused with <i>cccA'</i> -6xCAT, Amp ^R	This work
pVM4(P474)G9C	<i>mrpA</i> carrying mutation G9C, truncated at the codon encoding P474 and fused with <i>cccA'</i> -6xCAT, Amp ^R	This work
pMCh	<i>nuoM</i> fused with <i>cccA'</i> -6xCAT, Amp ^R	This work
pMMA(P474)	<i>mrpA</i> with first 4 codons replaced by first 4 codons of <i>nuoM</i> , truncated at the codon encoding P474 and fused with <i>cccA'</i> -6xCAT, Amp ^R	This work
pMMA(K679)	<i>mrpA</i> with first 4 codons replaced by first 4 codons of <i>nuoM</i> , truncated at the codon encoding K679 and fused with <i>cccA'</i> -6xCAT, Amp ^R	This work
pMMA(V734)	<i>mrpA</i> with first 4 codons replaced by first 4 codons of <i>nuoM</i> , truncated at the codon encoding V734 and fused with <i>cccA'</i> -6xCAT, Amp ^R	This work
pMMA	<i>mrpA</i> with first 4 codons replaced by first 4 codons of <i>nuoM</i> and fused with <i>cccA'</i> -6xCAT, Amp ^R	This work
pCW6	Cm ^R	Claes von Wachenfeldt
pVM11	<i>mrpA</i> fused with <i>cccA'</i> , Cm ^R	[18]
pMrpAtr	<i>mrpA</i> truncated at the codon encoding I472 and fused with <i>cccA'</i> , Cm ^R	This work
pVM7	<i>mrpABCD</i> fused with <i>cccA'</i> at <i>mrpD</i> , Cm ^R	[10]
pMrpAtrBCD	<i>mrpABCD</i> with <i>mrpA</i> truncated at the codon encoding I472 and fused with <i>cccA'</i> at <i>mrpD</i> , Cm ^R	This work
Primers		
MrpAtr-forw	5' P-GGGCTTGATGACTCTCGG 3'	
MrpAtrP474-rev	5' P-AGGCTCTATCAGGCTGTACGAC 3'	
MrpAtrK679-rev	5' P-TTTTGTTCAGCCTCAGTTTCG 3'	
MrpAtrV734-rev	5' P-GACATTGACGACATTATCCCCT 3'	
MrpAG9C-forw	5' P-AGAGTGCATACCGGCTGGTTTGT 3'	
MrpAG9C-rev	5' P-TTTTGCCTATTTTGCCAAGAAGG 3'	
MrpA-Blunt-T13 -forw	5' TGGTTTGTGTTGATCCTCG 3'	
Cytic-Blunt-G1 -forw	5' GGGCTTGATGACTCTCGGA 3'	
NuoM-Blunt-C 12-rev	5' P-GGGTAGTAACATGGCGATCTTTATTCCT 3'	
MrpA-pCW6-up	5' P-GGGCTTGATGACTCTCGGAGATTG 3'	
MrpA-pCW6-down	5' P-CTCTATCAGGCTGTACGACAATATATTCGGG 3'	
MrpABCD-pVM7-up	5' P-ACAGCATGATTAACAAAGTAAAGAGGAGGGGA 3'	
MrpABCD-pVM7-down	5' P-CTCTATCAGGCTGTACGACAATATATTCGGG 3'	

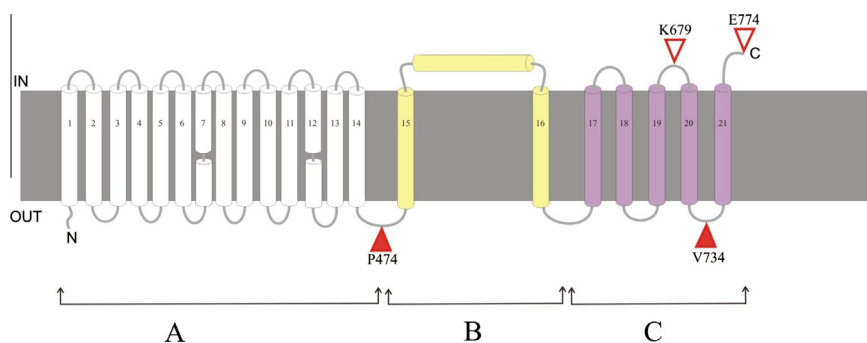


Fig. 1. Schematic drawing of the MrpA polypeptide with the fully conserved part (A), the part conserved only in NuoL (B, see also Fig. 2A) and the part conserved as NuoJ of complex I (C, see also Fig. 2B). The positions of the four cytochrome c_{550} fusions are indicated with triangles (see also Fig. 3), red, filled symbols for fusion proteins that express as holo-cytochrome and open symbols for apo-cytochrome.

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 EMBnet 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 c_{550} -His-tag [18]. The PCRs 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 c_{550} -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 *PpMUI* 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 MrpA-pCW6-up and MrpA-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* Δ MrpA 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 Amicol 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 manufacturer's instructions. The immunodetection was done as described previously [20] using anti-cyt c_{550} 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 c_{550} -tagged proteins were recorded using a Shimadzu UVPC 2100 spectrophotometer. Using the extinction coefficient of cytochrome $c_{550e} = 24 \text{ mM}^{-1}\text{cm}^{-1}$ [23], concentrations of the fusion proteins were calculated [20].

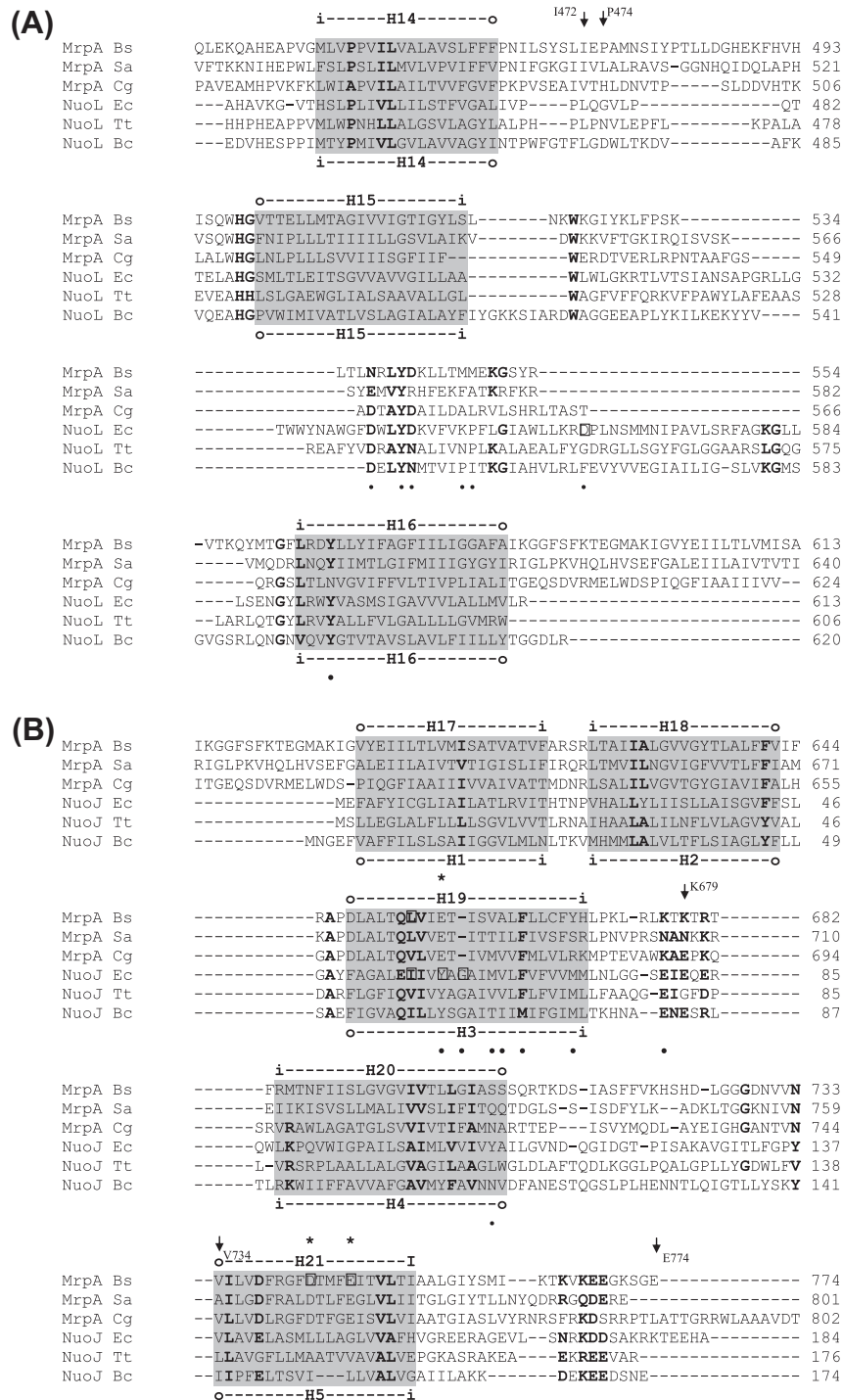


Fig. 2. A and B. Structure-based sequence alignments of the C-terminal domain of MrpA with NuoL (A) and NuoJ (B) (see also Fig. 1). The sequences are (from top to bottom): *Bacillus subtilis* [Q9K2S2], *Staphylococcus aureus* [QOQ2K0], *Corynebacterium glutamicum* [Q8NM51], *Escherichia coli* [P33607], *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 c_{550} 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.

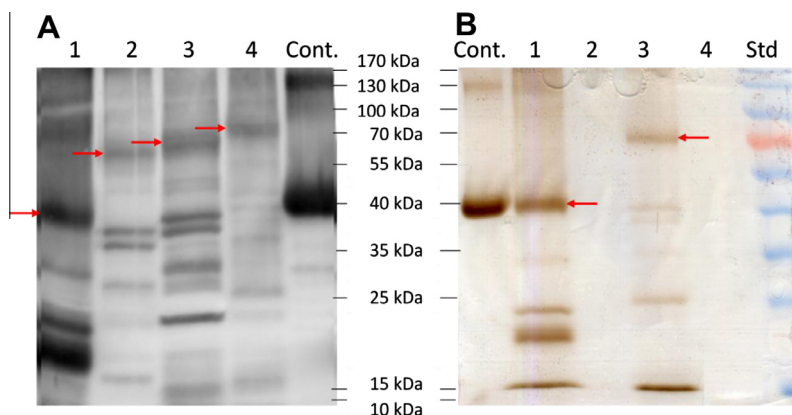


Fig. 3. The four different cytochrome c fusion proteins in purified and concentrated fractions detected by immunodetection with anti-cyt c_{550} antibodies (Panel A) and heme staining (Panel B). The calculated masses of the proteins are: 63.8 kDa for MMrpA(P474)cytH in Lane 1, 86.8 kDa for MMrpA(K679)cytH in Lane 2, 92.7 kDa for MMrpA(V734)cytH in Lane 3 and 97.1 kDa for MMrpAcytH 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 μ g of NuoMcytH, others contained 50 μ g of membrane protein. Both gels contain some degradation products however occurring only in the holo-cytochrome-proteins.

Table 2

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

<i>B. subtilis</i> Δ mrpA at 80 mM Na ⁺		
At pH 6.5	Max OD	g (min)
MrpA	1.72 \pm 0.03	71
MrpAtr	1.64 \pm 0.02	87
At pH 7.4		
MrpA	1.84 \pm 0.06	46
MrpAtr	1.49 \pm 0.05	97
MrpABCD	1.78 \pm 0.02	52
MrpAtrBCD	1.43 \pm 0.02	103
At pH 8.4		
MrpA	1.76 \pm 0.03	64
MrpAtr	0.17 \pm 0.02	na
MrpABCD	1.65 \pm 0.02	72
MrpAtrBCD	0.16 \pm 0.03	na

2.9. Growth studies in *B. subtilis*

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

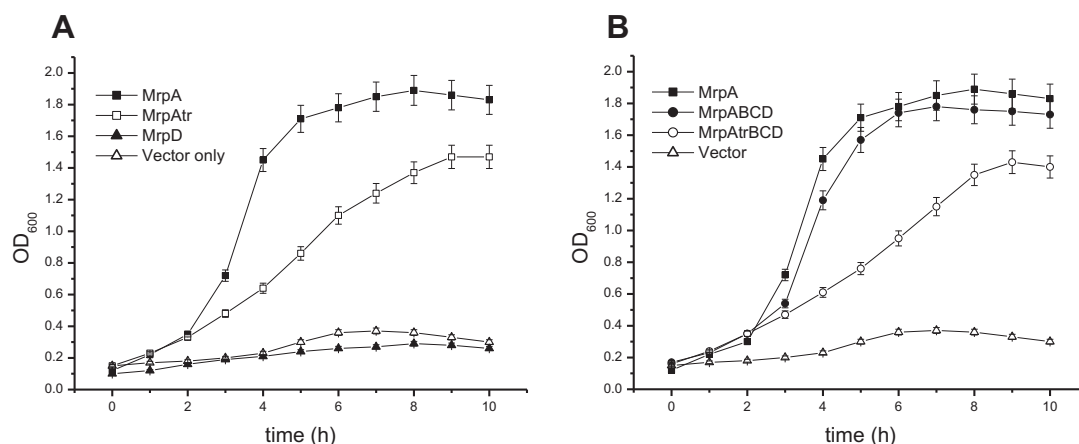


Fig. 4. Growth properties of the *B. subtilis* Δ mrpA deletion strain at pH 7.4 and 80 mM Na⁺. MrpA expressed *in trans* from a plasmid restores the wild type growth properties, but expression of MrpD does not improve the growth. Expression of MrpA lacking the C-terminal domain resulted in a moderate 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.

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

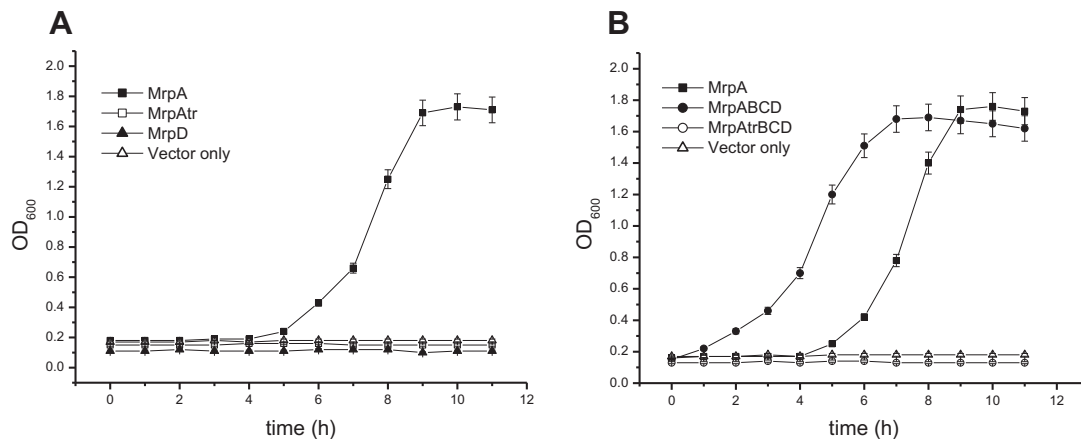


Fig. 5. Growth properties of the *B. subtilis* $\Delta mrpA$ deletion strain at pH 8.4 and 80 mM Na^+ . MrpA alone expressed *in trans* restores the wild type growth properties only after a lag-phase of about 4 h (A). Expression of the MrpABCD from a plasmid prevents the lag-phase (B), strongly suggesting that Mrp-complex formation is required for its function at pH 8.4. The truncated MrpA could not restore the growth, neither when expressed alone (A) nor when expressed together with MrpBCD (B). This implies that the C-terminal domain of MrpA is essential for Mrp-complex formation.

structural elements, corresponding to HLa plus HLB 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_{550} fusion method described previously [20]. Briefly, the heme *c* 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_{550} 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 made. Yet, the expressed amount of MMrpA(P474)cytH corresponding to the fully conserved part of MrpA (Fig. 1A), was only 30% of what we normally produce of Nuom. Still, it definitely contained a holo-cytochrome *c* (Fig. 3B) and just like all other fusion proteins could be detected by the antibodies (Fig. 3A). The full length MMrpAcytH protein did not contain heme in the polypeptide, neither did MMrpA(K679)cytH, whereas the MMrpA(V734)cytH contained heme (Fig. 3B). In crude fractions from cells expressing MMrpA(P474)cytH and MMrpA(V734)cytH, about 20 nmol of cytochrome *c* per mg membrane protein could be detected from optical spectra whereas membranes from cells expressing MMrpA(K679)cytH or full length MMrpAcytH did not contain any spectroscopically detectable heme. This confirms the predicted transmembrane topology of MrpA and its homology to the long horizontal helix and to Nuoj in complex I. The Mrp-complex contains an additional three small proteins, MrpE, F and G, of

poorly understood function [28], that do not seem to be conserved in complex I (but see also [29]). Such redundant proteins are easily lost during evolution, provided that they are not trapped in the protein for structural reasons.

Function of the C-terminal domain in MrpA. For synthesis in *B. subtilis*, the MrpA was expressed from the P_{SPAC} promoter of pCW6 but otherwise with its natural regulatory elements left in place. In these constructs (pMrpAtr and pMrpAtrBCD, see Table 1) MrpA

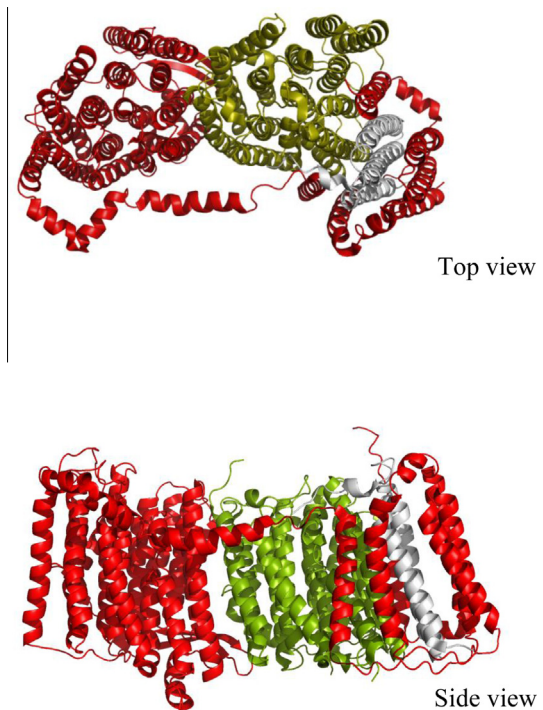


Fig. 6. Tentative model of the MrpA, C and D part of the Mrp-antiporter, based on the proposed structural similarities to complex I. In the model, the long horizontal helix has been truncated to correspond to the part conserved in MrpA, and Helix 16 in Nuol has been fused to Helix 1 in Nuoj to mimic the full length MrpA (red). Nuok is equivalent to MrpC (grey) and Nuon corresponds to MrpD (green). One of the large subunits, Nuom, has been removed from the complex I structure to resemble the Mrp-complex, with only two large antiporter-like polypeptides. The three Mrp subunits MrpE, F and G that are present in the full size Mrp-complex but not conserved in complex I, are not shown in the model.

was truncated at position I472 (Fig. 2A), both in the single MrpA subunit and in MrpABCD sub-complex, and expressed in *B. subtilis* Δ MrpA. 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* Δ MrpA 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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