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Involvement of the *Shewanella oneidensis* Decaheme Cytochrome MtrA in the Periplasmic Stability of the β-Barrel Protein MtrB▽

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The *Shewanella oneidensis* outer membrane β-barrel protein MtrB is part of a membrane-spanning protein complex (MtrABC) which is necessary for dissimilatory iron reduction. Quantitative PCR, heterologous gene expression, and mutant studies indicated that MtrA is required for periplasmic stability of MtrB. DegP depletion compensated for this MtrA dependence.

Dissimilatory iron reduction has been studied extensively since it was discovered in the 1980s to be a microbial respiratory process (11, 13–15). The physiological challenge of this form of respiration is that environmentally relevant ferric iron forms at neutral pH are crystalline iron oxi(hydroxi)des (19). Therefore, dissimilatory iron reduction necessitates an extended respiratory chain through the periplasm and the outer membrane to access the insoluble electron acceptor.

Recently, it was shown that an outer membrane protein complex in *Shewanella oneidensis* is capable of catalyzing electron transfer over a liposomal membrane (7) and hence *in vivo* most probably over the outer membrane. This complex consists of the periplasmic *c*-type cytochrome MtrA, the outer membrane β-barrel protein MtrB, and the outer membrane *c*-type cytochrome MtrC (7, 17). Mutants in any of these three proteins are either strongly affected in their ability or unable to use ferric iron as the sole electron acceptor (1).

A puzzling phenotype was recently described whereby in an *mtrA* deletion mutant MtrB could not be detected (7). However, the mechanism of this dependence is not known. This study aims to elucidate the reason for this MtrA dependence for the formation of MtrB.

MtrA is not necessary for MtrB transcription. *mtrA* and *mtrB* are adjacent genes carried in the same operon. The dependence of MtrA for MtrB production could be due to regulatory elements within the *mtrA* gene. Hence, we determined whether deletion of *mtrA* affects the quantity of *mtrB* transcripts. Independent triplicates of wild-type *S. oneidensis* and a markerless *mtrA* deletion mutant (20) were grown in minimal medium containing 100 mM fumarate as the electron acceptor and 50 mM lactate as the electron and carbon source. RNA was isolated using the RNeasy minikit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. *recA*, *gyrB*, and *dnaK* were selected as internal reference genes for normalization of the expression ratio of *mtrB* (Table 1). Fumarate was chosen as the electron acceptor since *mtrA* mutants are unable to grow under Fe(III)-reducing conditions. Furthermore, the proteomes of chelated iron- and fumarate-grown cells are very similar (18). Transcript quantification revealed that wild-type cells contain a 1.24 ± 0.059-fold higher number of *mtrB* transcripts than the Δ*mtrA* mutant. Only a more than 1.5-fold change in the gene expression ratio is biologically significant (6). Therefore, *mtrB* is transcribed in almost equal amounts in both strains, and MtrA does not seem to be necessary for *mtrB* transcription or mRNA stability.

Heterologous *mtrA*/*mtrB* expression in *Escherichia coli*. *mtrA* and *mtrB* were expressed in *E. coli* to determine whether the same pattern of MtrA-dependent MtrB production would be detectable. This would suggest that general factors for export, maturation, or localization of β-barrel proteins in Gram-negative bacteria are causative for the observed MtrA-dependent MtrB formation.

E. coli BL21(DE3) was chosen as a host for T7 polymerase-

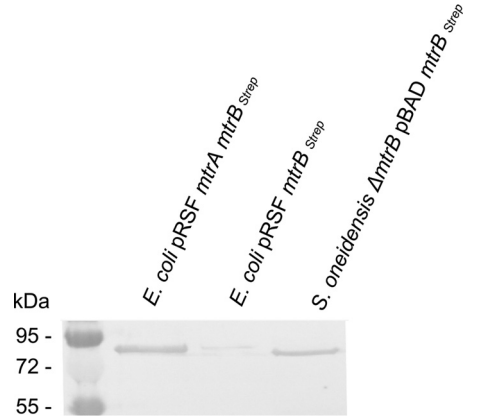

FIG. 1. Western blot of membrane fractions from *E. coli* BL21 pRSF *mtrA* $mtrB_{Strep}$ and *E. coli* BL21 pRSF $mtrB_{Strep}$ strains grown under fermentative conditions in the presence of 50 μM IPTG. A membrane fraction of a Δ*mtrB* mutant strain complemented with plasmid pBAD $mtrB_{Strep}$ was used as the positive control. Two micrograms was loaded from each *E. coli* membrane fraction, while 50 μg was loaded from the positive control.

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TABLE 1. Primer used in this work

Primer no.	Primer name	Sequence	Usage[a]
1	BspHI_recA_for	CGGCGGTCATGAGTATCGACGAAAACAAACAG	Construction of pKD46 *recA*
2	HindIII_recA_rev	GCCAAGCTTTTAAAAATCTTCGTTAGTTTC	Construction of pKD46 *recA*
3	pKD46_exo_int_for	GAGGCACTGGCTGAAATTGG	Sequencing of pKD46 *recA*
4	recA_int_rev	GTTTACGTGCGTAGATTGGG	Sequencing of pKD46 *recA*
5	mtrB_NcoI_for	CATGCCATGGATGAAATTTAAACTCAATTT	Construction of pBAD $mtrB_{Strep}$
6	mtrB_strep_HindIII_rev	GGGAAGCTTTTATTTTTCGAACTGCGGGTGGCTCCAGGC GCCGAGTTTGTAACTCATGCT	Construction of pBAD $mtrB_{Strep}$
7	ΔmtrA_up_for	GATCCCCGGGTACCGAGCTCGAATTCGTAACATTCCCAG CGGTCGGTC	*mtrA* knockout mutant
8	ΔmtrA_up_rev	AATAGGCTTCCCAATTTGTCCC	*mtrA* knockout mutant
9	ΔmtrA_down_for	CGAATTCTGGGACAAATTGGGAAGCCTATTCAGCGCTAA GGAGACGAG	*mtrA* knockout mutant
10	ΔmtrA_down_rev	AGCTTGCATGCCTGCAGGTCGACTCTAGAGGTTCGAGGG CATTGAGGC	*mtrA* knockout mutant
11	mtrBgen_up_for	ATGATTACGAATTCGAGCTCGGTACCCGGGGGGGTGGCG GATGAACTGTC	Strep epitope knock-in
12	mtrBgen_up_rev	GGCGCCTGGAGCCACCCGCAGTTCGAAAAATAATCCATT TGCCTCATATGCTC	Strep epitope knock-in
13	mtrBgen_down_for	TTATTTTTCGAACTGCGGGTGGCTCCAGGCGCCGAGTTT GTAACTCATGCT	Strep epitope knock-in
14	mtrBgen_down_rev	CGGCCAGTGCCAAGCTTGCATGCCTGCAGGCAAAAGACA CCAGTTATGATG	Strep epitope knock-in
15	ΔdegP_up_for	ATGATTACGAATTCGAGCTCGGTACCCGGGCCATCTCGA GTAAGATCTTTTTG	*degP* knockout mutant
16	ΔdegP_up_rev	CTATTCATAACTCCAAATAAGGG	*degP* knockout mutant
17	ΔdegP_down_for	CTTATTTGGAGTTATGAATAGTCAATTGGCGAATCTGATC	*degP* knockout mutant
18	ΔdegP_down_rev	CGGCCAGTGCCAAGCTTGCATGCCTGCAGGCACTTCAGA GGTGAACTTGC	*degP* knockout mutant
19	RT_mtrB_for	CGGCTTAAAACAAGCCTCTG	qRT-PCR
20	RT_mtrB_rev	CCAAAGGTGGGGTTAAAAGC	qRT-PCR
21	dnaK_for	ATGGGTAAAATTATTGGTATC	qRT-PCR
22	dnaK_rev	TTATTTCTTGTCGTCTTTCAC	qRT-PCR
23	RT_dnaK_for	CGTGACGTGAACATCATGC	qRT-PCR
24	RT_dnaK_rev	CAGAAACCTGTGGTGGAGC	qRT-PCR
25	RT_gyrB_for	GCTTGATTGAAGTCGGTGGT	qRT-PCR
26	RT_gyrB_rev	CGTTTCGCTTCAGAAATGGT	qRT-PCR
27	RT_recA_for	AGCTATAGCCGCTGAAATCG	qRT-PCR
28	RT_recA_rev	CCTCGACATTGTCATCATCG	qRT-PCR
29	HindIII_mtrA_rev	GGGAAGCTTTTAGCGCTGTAATAGCTTGC	Construction of pRSF *mtrA* $mtrB_{Strep}$
30	BspHI_mtrA_for	GAAATATCATGAAGAACTGCCTAAAAATG	Construction of pRSF *mtrA* $mtrB_{Strep}$
31	mtrB_NdeI_for	GGAATTCCATATGAAATTTAAACTCAATTTGATC	Construction of pRSF $mtrB_{Strep}$
32	KpnI_mtrBstrep_rev	CGGGGTACCTTATTTTTCGAACTGCGGGTGGCTCCAGGC GCCGAGTTTGTAACTCATGCT	Construction of pRSF $mtrB_{Strep}$
33	pRSF_MCS1_for	GGATCTCGACGCTCTCCCT	Sequencing of pRSF *mtrA* $mtrB_{Strep}$
34	pRSF_MCS2_for	TTGTACACGGCCGCATAATC	Sequencing of pRSF $mtrB_{Strep}$
35	BL21_ΔdegP_ rev	CAGATTGTAAGGAGAACCCCTTCCCGTTTTCAGGAAGGG GTTGAGGGAGACTAAGCACTTGTCTCCTGTTT	Construction of conditional *degP* mutant
36	E.coli_degPtet_for	TTTGTAAAGACGAACAATAAATTTTTACCTTTTGCAGAAA CTTTAGTTCGTTTAAGACCCACTTTCACATTTAA	Construction of conditional *degP* mutant

[a] qRT-PCR, quantitative reverse transcription-PCR.

dependent expression of either $mtrB_{Strep}$ alone or *mtrA* and $mtrB_{Strep}$ using the pRSFDuet-1 expression plasmid (Table 1) (Merck, Darmstadt, Germany). The *mtrB* gene was modified to contain a Strep-tag epitope to allow for subsequent immunodetection (Table 1). *E. coli* strains containing either pRSF $mtrB_{Strep}$ or pRSF *mtrA* $mtrB_{Strep}$ were grown in LB medium to an optical density at 600 nm (OD_{600}) of 0.3. Subsequently, the cell suspension was transferred to glass bottles and sealed with rubber stoppers to proceed growth under fermentative conditions. Expression of the T7 polymerase gene was induced with 50 μM IPTG (isopropyl-β-D-thiogalactopyranoside), and growth was continued at room temperature for 10 h. MtrB was detected by Western blot analysis using a primary antibody specific for the attached Strep-tag epitope (Qiagen, Hilden, Germany).

Interestingly, MtrB was not, or only faintly, detectable in membrane fractions when produced without concurrent expression of *mtrA* even when expressed in *E. coli* BL21 (Fig. 1). In contrast, MtrA coexpression resulted in a strongly detectable production of MtrB.

Multidimensional protein identification technology (MudPIT) analysis of periplasmic protein fractions from *S. oneidensis*. Having shown that identical patterns of dependence of MtrB production on MtrA expression is present in *E. coli* and *S. oneidensis*, we screened for periplasmic proteases in *S. oneidensis*

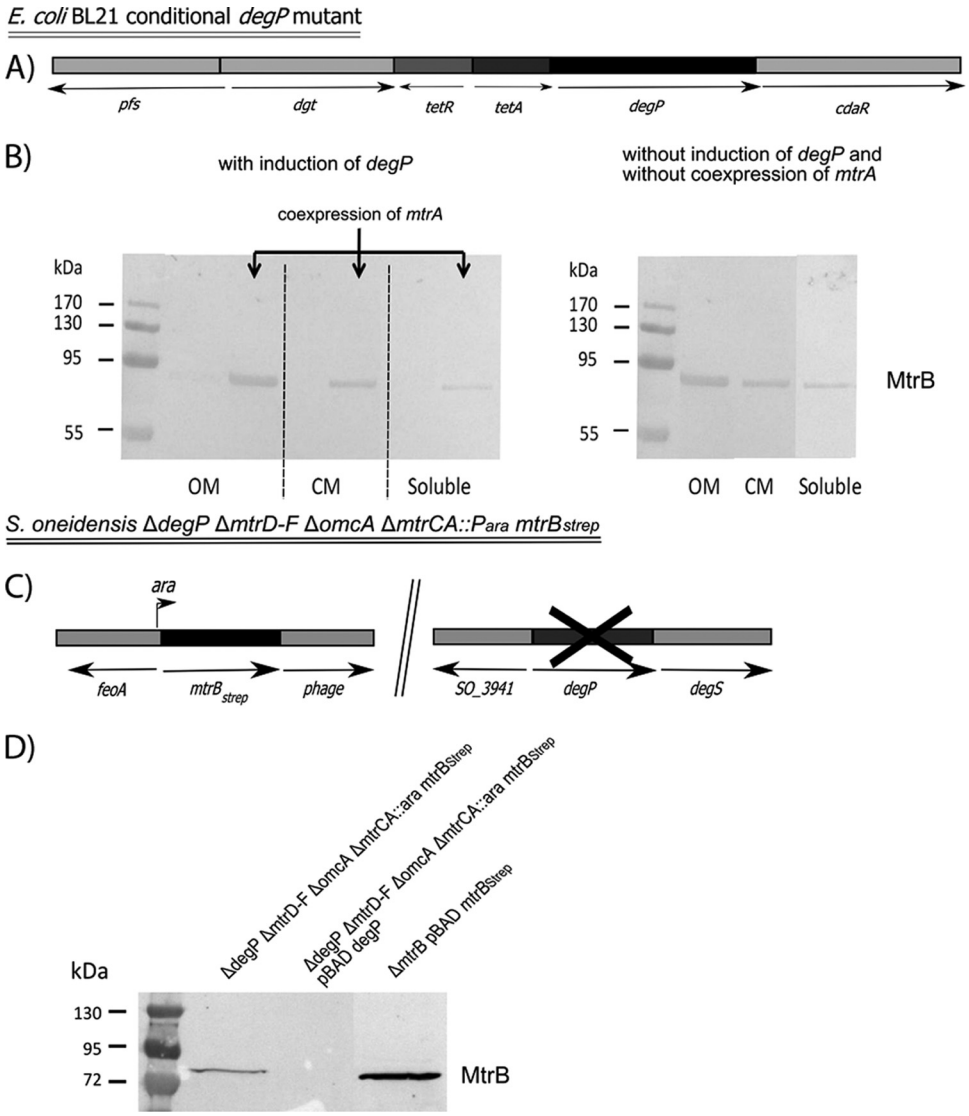

FIG. 2. (A) Relevant genotype of the *E. coli* BL21 conditional *degP* mutant. Heterologous expression of $mtrB_{Strep}$ or *mtrA* $mtrB_{Strep}$ was achieved using either plasmid pRSF $mtrB_{Strep}$ or pRSF *mtrA* $mtrB_{Strep}$ and the concomitant addition of 50 μM IPTG. (B) Left, Western blot of protein fractions from *E. coli* pRSF $mtrB_{Strep}$ and *E. coli* pRSF *mtrA* $mtrB_{Strep}$ strains. *degP* expression was induced with 0.2 mg ml^{-1}anhydrotetracycline. Right, Western blot of protein fractions from the *E. coli* pRSF $mtrB_{Strep}$ strain. The induction of *degP* was omitted in this experiment. Two micrograms from each protein fraction was loaded on the gel. OM, outer membrane fraction; CM, cytoplasmic membrane fraction; Soluble, soluble protein fraction. (C) Relevant genotype of the *S. oneidensis* MR-1 Δ*degP* Δ*mtrD* Δ*mtrE* Δ*mtrF* Δ*omcA* Δ*mtrCA*::P_{ara} $mtrB_{Strep}$ strain. (D) Corresponding Western blot of outer membrane protein fractions derived from *S. oneidensis* MR-1 Δ*degP* Δ*mtrD* Δ*mtrE* Δ*mtrF* Δ*omcA* Δ*mtrCA*::P_{ara} $mtrB_{Strep}$ cells or its *degP* complemented version grown under anaerobic conditions with fumarate as the terminal electron acceptor. A membrane fraction of a complemented Δ*mtrB* mutant strain was used as the positive control. Fifty micrograms of each protein fraction was loaded on the gel.

that could affect MtrB stability and that are similar to periplasmic proteases of *E. coli* (2).

Using MudPIT mass spectrometry, 30 μg of a periplasmic protein fraction of *S. oneidensis* grown under ferric iron-reducing conditions was analyzed. Tryptic digestion followed by an automated 17-step, 34-hour MudPIT program on a linear ion-trap mass spectrometer (LTQ-FT-ICR; Thermo Scientific) was carried out for analysis as previously described (8, 22, 23).

Two proteases with a typical *sec* leader sequence for export into the periplasm were detected. One of these (SO_3942 [score, 93.13; coverage, 37.6%; 9 peptides detected]) is annotated as a serine protease of the HtrA/DegQ/DegS family, while the other (SO_3411 [score, 15.03; coverage, 3.9%; 2 peptides detected]) is annotated as a putative protease. DegP-dependent protein hydrolysis is ubiquitously distributed in Gram-negative bacteria. It is necessary for the degradation of misfolded outer membrane proteins (9). In *S. oneidensis*, the SO_3942 gene is located directly upstream of *degS*. DegS is involved in the σ^E-dependent stress response which results in upregulation of *degP* (2). BLAST analysis of SO_3942 revealed a very high similarity to DegP from *E. coli* (score, 447; E value,

7e−127). Due to this similarity and clustering with *degS*, we will now refer to *S. oneidensis* SO_3942 as DegP.

Influence of *E. coli degP* expression on MtrB stability. If DegP degrades MtrB in the absence of MtrA, then it should be possible to uncouple this MtrA dependence in the absence of DegP. Following the method described by Datsenko and Wanner (5), a conditional *degP* mutant in *E. coli* BL21 was constructed via integration of a tetracycline resistance cassette in place of the native *degP* promoter, allowing for *tet* promoter control of *degP* (Fig. 2A; Table 1). Strains expressing either $mtrB_{Strep}$ or *mtrA* and $mtrB_{Strep}$ in a conditional *degP* mutant background were grown as described above. Each of the samples was split into duplicates, and *degP* expression was induced in half of the flasks via the addition of 0.2 $\mu g\ ml^{-1}$ anhydrotetracycline. Upon induction of *degP*, MtrB again was detected only when *mtrA* was coexpressed (Fig. 2B). In contrast, MtrB was detectable even without concurrent expression of MtrA in the absence of *degP* expression. Moreover, MtrB was, for the most part, correctly localized to the outer membrane and detectable only to a minor extent in the cytoplasmic membrane fraction or the soluble protein pool (Fig. 2B). Membrane separation was achieved using the method described by Leisman et al. (10).

Uncoupling of the MtrA/MtrB dependence in *S. oneidensis*. Finally, we tested whether the same influence of DegP on MtrB stability would also be detectable in *S. oneidensis*. A markerless *degP* deletion mutant (Fig. 2C) was constructed in the *S. oneidensis* Δ*mtrD* Δ*mtrE* Δ*mtrF* Δ*omcA* Δ*mtrCA*::P_{ara} $mtrB_{Strep}$ strain (3) according to the method described by Schuetz et al. (20). In this strain, $mtrB_{Strep}$ is under pBAD promoter control and contains an extension coding for the Strep-tag epitope (Table 1). As expected, MtrB was detectable in the outer membrane after induction with 1 mM arabinose. Complementation of the *degP* deletion by *degP* in *trans* led to the loss of the MtrB signal, which confirms the results obtained in *E. coli* (Table 1; Fig. 2D).

Implications. In this study, we provide evidence for a dual function of the periplasmic *c*-type cytochrome MtrA. The high heme content and its localization in the periplasm as well as at the outer membrane clearly point toward a role in electron transfer (16, 20). Furthermore, our experiments show that the periplasmic presence of MtrA seems to be necessary for resistance against DegP-based degradation of MtrB. Of note, DegP was shown to form large oligomeric complexes that might be involved in the sequestering of misfolded proteins (4, 12). Still, MtrB was not detectable in the presence of DegP in an *mtrA* deletion mutant. Therefore, unfolded MtrB does not seem to be sequestered but rather degraded by DegP.

It was shown that β-barrel proteins are usually guided through the periplasm via the interaction with chaperones like SurA or Skp (21). Binding of these chaperones prevents protease hydrolysis. One hypothesis regarding the function of MtrA in MtrB stability could be that MtrA itself binds to unfolded MtrB and may have a scaffold-like function. It was shown that MtrA forms a stable complex with MtrB at the outer membrane (7). Formation of this complex could be initiated in the periplasm when MtrB is in an unfolded state. Efforts to identify periplasmic interactions of soluble MtrB with MtrA and/or other proteins *in vivo* have been thus far unsuccessful. Nevertheless, this will clearly be a future direction of our research.

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